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DOCTORAL DISSERTATION

Cytochrome P450 1A1 and Aromatase (CYP19) in Cetaceans: Enzyme Expression and Relationship to Contaminant Exposure

by

Joanna Yvonne Wilson

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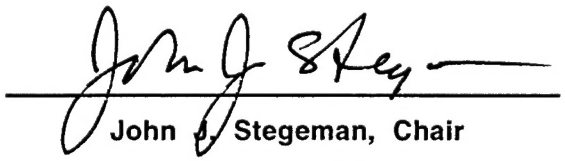

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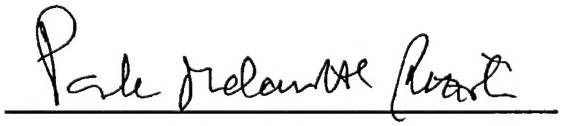

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by

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Submitted in partial fulfillment of the requirements for the degree of

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at the

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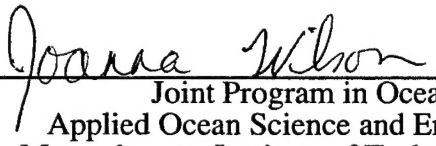
Signature of Author 
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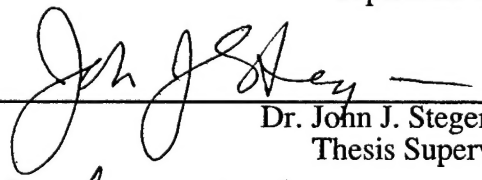
Certified by 
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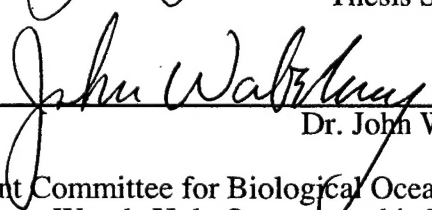
Accepted by 
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Cytochrome P450 1A1 and Aromatase (CYP19) in Cetaceans:

Enzyme Expression and Relationship to Contaminant Exposure

by

Joanna Yvonne Wilson

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Contaminant exposure has been associated with toxic effects in marine mammals. Studies on early biochemical and molecular responses prior to, but associated with, contaminant-induced toxicity will aid in understanding the susceptibility of cetaceans to such exposures. Cytochrome P450 1A1 (CYP1A1) induction by planar halogenated aromatic hydrocarbons, including certain polychlorinated biphenyls (PCBs), has been correlated with toxic effects in rodents. CYP1A1 expression was measured in multiple organs from beluga whales and white-sided dolphins and integument biopsies from bottlenose dolphins. Age and sex do not appear to influence CYP1A1 expression in these species. Mono-*ortho* PCB concentrations were correlated with CYP1A1 in bottlenose dolphin integument and white-sided dolphin liver. White-sided dolphins, with PCBs concentrations six times higher than Arctic beluga, have lower hepatic CYP1A1 expression. Beluga whales thus appear very sensitive to the induction of CYP1A1 by PHAHs. These studies demonstrate that comparative studies between cetacean species, with reference to experimental work in model organisms, could determine the relative susceptibility of cetaceans to contaminant exposures.

Aromatase is the enzyme responsible for estrogen production and recent studies suggest that PCBs may alter its activity or expression. We determined the sequence and characterized the catalytic properties of a cetacean aromatase, which was similar to other mammalian aromatases. Evolutionary analyses of vertebrate aromatases revealed striking functional conservation. Although much work is required, these data show that similar to CYP1A1, aromatase studies in other mammals are applicable to cetacean species. Experimental work in model organisms, coupled with careful studies with cetacean tissues, could infer the physiological role and transcriptional control of aromatase in cetacean species.

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This thesis is dedicated, with love and gratitude, to Andrew and Galen McArthur

I have seen
A curious child, who dwelt upon a tract
Of inland ground, applying to his ear
The convolutions of a smooth-lipped shell;
To which, in silence hushed, his very soul
Listened intensely; and his countenance soon
Brightened with joy; for from within were heard
Murmurings, whereby the monitor expressed
Mysterious union with its native sea.

- William Wordsworth, *Excursion* (bk. IV)

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ABBREVIATIONS

AHR	aryl hydrocarbon receptor
ArKO	aromatase knockout
ARNT	aryl hydrocarbon receptor nuclear transferase
BMPO	benzo(a)pyrene monooxygenase
βNF	β-napthoflavone
CB	chlorobiphenyl
CYP	cytochrome P450
DDT	o,p'-dichlorodiphenyltrichloroethane
DilAC-LDL	dil-acetylated low density lipoprotein
DMBA	7,12-dimethylbenz[a]anthracene
DRE	dioxin response element
ED50	dose causing half-maximal response of test animals
ER	estrogen receptor
7-ER	7-ethoxyresorufin
EROD	ethoxyresorufin-O-deethylase
LD50	lethal dose with 50% mortality of test animals
HCB	hexachlorobenzene
HCH	hexachlorocyclohexane
7-MR	7-methoxyresorufin
MROD	methoxyresorufin-O-demethylase
NIST	National Institute of Standards and Technology
PBDE	polybrominated diphenyl ethers
PAH	polycyclic aromatic hydrocarbons
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PHAH	planar halogenated aromatic hydrocarbons
ROS	reactive oxygen species
SLE	St. Lawrence Estuary

TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran
XRE	xenobiotic response element

Chapter 1. Introduction

Planar halogenated aromatic hydrocarbons (PHAHs), including specific polychlorinated biphenyl (PCB), polychlorinated dibenzo-p-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF) congeners, have a wide range of effects in mammals. At high doses, PHAHs cause lethality, wasting, lymphoid and gonadal atrophy, chloracne, hepatotoxicity, neurotoxicity, and cardiotoxicity (Birnbaum and Tuomisto 2000). At lower doses, PHAHs adversely affect the immune system, development and reproduction (Birnbaum *et al.* 2000). The lethal dose causing 50% mortality (LD_{50}) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent PHAH, varies by over 1000-fold between guinea pigs and hamsters. Although this degree of species difference is not as extreme in other endpoints, this example highlights the potential for very different sensitivities to PHAHs in closely related organisms. Species differences prevent the direct extrapolation of data in standard test organisms to possible effects in wildlife species, based on contaminant concentrations alone. Yet, controlled experimental exposures are precluded in cetacean species because of logistical and ethical issues. Thus, assessing biological responses in both field samples and with *in vitro* experiments are perhaps the only ways to determine the sensitivity of cetacean species to PHAH toxicity.

Contaminants of Concern

For cetaceans, organochlorine compounds including chlorinated pesticides and planar halogenated aromatic hydrocarbons (PHAHs) have traditionally been compounds of concern. The polychlorinated biphenyls are a group of 209 congeners with varying degrees of chlorination that were originally used as placticizers, adhesives and dielectric fluids in capacitors and transformers. These compounds are currently banned in most countries and yet they persist in the environment. The most persistent PCBs with multiple sites of chlorination are those contaminants measured most typically in cetacean blubber. PCB congeners (Figure 1) are identified by the location of chlorine substitution and those important for cytochrome P450 1A1 induction (discussed below) either lack or have only a single *ortho* substitution (2, 2', 6, or 6' positions). Also included in the PHAHs are the laterally substituted PCDDs and PCDFs (Figure 1), although the

concentrations of these contaminants are low in cetacean blubber (O'Shea and Brownell 1994).

The polycyclic aromatic hydrocarbons (PAHs) are key products of combustion and are rarely measured in cetacean tissues because they are readily metabolized and do not bioaccumulate, although at least one study has shown they are measurable in blubber (Marsili *et al.* 2001). Although many PAHs with greater than three rings, such as benzo(a)pyrene (Figure 1), have the potential to induce CYP1A1 in a manner similar to PHAHs, their relative contribution to chemical exposure in cetaceans is not well understood because of the dearth of PAH measurements in cetacean tissues. Their importance in affecting marine organisms remains relatively unknown and yet, due to their ability to induce CYP1A1, they are considered in the scope of this thesis.

Other chlorinated and brominated compounds persist in the environment and have chemical characteristics such as a high lipophilicity that allow them to accumulate in fatty tissues similar to PHAHs. These recalcitrant compounds, which are not easily metabolized and/or excreted, include the chlorinated pesticides and polybrominated diphenyl ethers (flame retardants, PBDEs, Figure 1). Although these compounds may cause different toxic effects than the PHAHs and PAHs, they accumulate in cetacean blubber and are often measured in contaminant burden studies. The chlorinated pesticides most commonly measured in cetaceans include DDT (Figure 1) and its metabolites, hexachlorobenzene (HCB), hexachlorocyclohexanes (HCH), dieldrin, endrin, mirex, nonachlor, and toxaphene.

Contaminant Burdens in Cetaceans

Cetaceans accumulate liphophilic, persistent contaminants in their blubber; the main internal fat store in cetaceans (Lockyer, C. 1986; Pond and Mattacks, 1987). Contaminants, such as PCBs and organochlorine pesticides, are resistant to *in vivo* metabolism and/or environmental degradation and biomagnify through the food chain. Since cetaceans are near or at the top of the marine food chain, contaminants can accumulate to very high concentrations. Total PCBs and ΣDDT (DDT plus its metabolites DDE and DDD) (Figure 1) have been recorded at concentrations over 1000

and 250 ppm in cetacean blubber (wet weight), respectively. The highest concentrations of PCBs have been found in striped dolphins from the Mediterranean Sea (Aguilar and Borrell 1994), beluga whale from the St. Lawrence Estuary (Muir *et al.* 1996) and killer whales from the Pacific Northwest (Ross *et al.* 2000). The maximum concentrations of contaminants found in odontocetes (toothed whales) are orders of magnitude higher than those typically reported in mysticetes (baleen whales) and at least one order of magnitude higher than the maximum concentration reported for a mysticete (O'Shea *et al.* 1994). Odontocetes presumably have higher concentrations of contaminants than mysticetes because they feed on higher trophic levels. In general, mysticetes have less than 5 ppm (wet weight basis) of either type of contaminants (O'Shea *et al.* 1994). Among odontocetes, ΣDDT concentrations in blubber ranged from 1 - 250 ppm while total PCBs concentrations range from 1 ppm - 1000 ppm. Despite these wide ranges, concentrations of either contaminant in odontocetes typically are not greater than 60 ppm.

Effects of Contaminants in Marine Mammals

Although marine mammals accumulate high concentrations of contaminants, the link between PHAH exposure and effects is difficult to establish. Limited data suggest that PHAHs could have a role in immunotoxicity, pathologies such as tumors, and reproductive difficulties of some marine mammal populations. Much of the information collected is on seals and sea lions (i.e. pinnipeds and otarids) and studies on the possible effects in cetaceans are limited. Most of these studies are observations of disease in populations of marine mammals exposed to high concentrations of contaminants in the environment, and do not establish cause and effect relationships. Controlled, experimental exposures are very rarely possible in marine mammals generally, and are impossible in cetacean species.

Current data suggest that organochlorine contaminants, including PHAHs, may cause immunotoxicity in wild marine mammal populations. High organochlorine concentrations have been noted in several marine mammal populations that have suffered high mortalities from epizootic infections (Hall *et al.* 1992; Aguilar *et al.* 1994; Kuiken *et al.* 1994; Lipscomb *et al.* 1994; Guitart *et al.* 1996; Birkun *et al.* 1999; Kajiwara *et al.*

2002). In these studies, animals that died from infections had higher contaminant concentrations than the concentrations found in animals that survived, or in populations that were not subject to large mortality. These studies have suggested that organochlorines, including PCBs, are immunotoxic to wild marine mammals. Captive field studies in harbour seal have linked organochlorines and decreased immune function (reviewed in Ross *et al.* 1996), but our understanding of the immunotoxic effects of PHAHs in cetaceans is less clear. High levels of PCBs were found in dead dolphins sampled during morbillivirus-related mass mortality events (Kuehl *et al.* 1994; Guitart *et al.* 1996; Birkun *et al.* 1999), suggesting that organochlorines are also immunotoxic in cetaceans. Decreased lymphocyte responses in peripheral blood have been related to organochlorine concentrations in free-ranging bottlenose dolphin (Lahvis *et al.* 1995). PCB concentrations were higher in stranded harbor porpoises that died from infectious disease than in harbor porpoises that died from physical trauma (Kuiken *et al.* 1994; Jepson *et al.* 1999). Collectively, these studies correlated high contaminant exposures with mortality caused by epizootic infections. The implication is that contaminants are immunotoxic and thus, animals with higher concentrations are more likely to die from infections.

The St. Lawrence beluga whale population offers the clearest evidence for a link between contaminants and pathology in cetaceans. This beluga population, a mere 500 individuals, accounts for 39% of all tumors reported in cetaceans (De Guise *et al.* 1994). The St. Lawrence beluga have high contaminant concentrations in blubber and suffer from an unusual prevalence of tumors as well as multi-systemic and viral lesions not seen in other cetacean populations (Martineau *et al.* 1988). These tumors are particularly prevalent in the digestive tract, accounting for 30% of tumors found in beluga. Tumors were the primary cause of death in 18% of the animals that have been examined (Martineau *et al.* 2002). Both the location and rate of tumors is very unusual for a cetacean population (Martineau *et al.* 2002). The annual tumor rate of the St. Lawrence beluga is similar to or higher than that recorded for humans and domesticated animals that receive veterinary care; groups that should have higher tumor rates than wild populations because tumors are age related and free-ranging animals have shorter

lifespans than those with medical care (Martineau *et al.* 2002). The finding of unusually high concentrations of contaminants in a population of animals that suffer from an unusual rate and types of tumor strongly link contaminant exposure and tumors in marine mammals.

While our knowledge of the effects of PHAHs on reproduction is very limited, higher PCB concentrations have been associated with lower pup production in a harbor seal population from the Dutch Wadden Sea (Reijnders 1980). An experimental exposure of captive common seals has demonstrated that higher PCB exposures cause reduced pup production related to lower levels of estradiol and implantation difficulties (Reijnders 1986). Additionally, altered testosterone concentrations have been correlated with organchlorine concentrations in Dall's porpoise (Subramanian *et al.* 1987). Although there is little direct evidence of PHAH induced toxicity in marine mammal species, the indirect evidence from the field studies cited above strongly suggest that it may occur in some populations.

Cytochrome P450 Enzymes in Cetaceans

Cytochrome P450 1A (CYP1A) induction is a biochemical change that is widely used as a biomarker for exposure of vertebrates to PHAHs. Induction of CYP1A also may be used as a biomarker for effects of the more potent PHAHs, as the toxicity of PHAHs, including TCDD, correlates with their ability to induce CYP1A (Poland and Knutson 1982; Safe 1986). CYP1A is a member of the cytochrome P450 superfamily of enzymes that catalyze the metabolism of a wide range of both xenobiotics and endogenous compounds in both eukaryote and prokaryote species (Nelson *et al.* 1996).

Cytochrome P450 (CYP) proteins have been identified in cetacean tissues in only a few studies (Goksoyr *et al.* 1985; Goksoyr *et al.* 1988; Watanabe *et al.* 1989; White *et al.* 1994; White *et al.* 2000) and inferred by congener-specific analysis of contaminant profiles in the blubber of cetaceans (Tanabe *et al.* 1988; Duinker *et al.* 1989; Norstrom *et al.* 1992). Congener-specific analysis of contaminant profiles in blubber is an indirect method of estimating CYP function that has been used to infer that cetaceans have CYP1A but little CYP2B-like enzyme activity (Tanabe *et al.* 1988; Norstrom *et al.*

1992). Catalytic assays and immunoblots showed that at least one CYP1A enzyme exists in striped dolphins, and minke, beluga, pilot, sperm and killer whales (Goksoeyr *et al.* 1988; Watanabe *et al.* 1989; White *et al.* 1994; White *et al.* 2000; Boon *et al.* 2001). CYP2B-like proteins have been found in minke, beluga and pilot whale, although a CYP2B1-like protein may not be present in cetaceans (Goksoeyr *et al.* 1988; White *et al.* 1994; White *et al.* 2000; Boon *et al.* 2001). CYP2E1-like, 3A-like and 4A-like proteins are also expressed in some species (Watanabe *et al.* 1989; White *et al.* 1994; Boon *et al.* 2001). All of the studies outlined above with direct measurements of cytochrome P450 were performed using liver microsomes. Antibodies for CYP1A or CYP2B did not cross-react with proteins in minke whale kidney microsomes (Goksoeyr *et al.* 1988). Therefore, it appears that there are multiple cytochrome P450 enzymes, including a CYP1A1 protein, in cetacean species and microsome studies show they are expressed in the liver. Although the presence of multiple cytochrome P450 proteins in extrahepatic organs is presumed, extrahepatic expression has been demonstrated with CYP3A4 (Celander *et al.* 2000) and CYP1A1 (Stegeman *et al.* 1994) antibodies in pilot whale. The identity and the degree of expression of different cytochrome P450 proteins remain unclear in cetaceans.

CYP1A in Mammals

In the CYP1A gene family, CYP1A1 and CYP1A2 are expressed in mammals (Nelson *et al.* 1996). CYP1A1 has been sequenced from several cetacean species (C. Godard, unpublished data and Teramitsu *et al.* 2000). In humans, CYP1A2 expression is restricted to liver and olfactory while CYP1A1 is expressed in both hepatic and extra-hepatic tissues. CYP1A enzymes are inducible by PHAHs and PAHs and this induction typically increases the metabolism of the inducer (Whitlock 1999). Hepatic CYP1A1 expression has been correlated to the concentrations of mono-*ortho* PCBs in blubber of beluga whale (White *et al.* 1994). CYP1A1 expression in skin has been induced by dietary exposure to oil in otters (Ben-David *et al.* 2001). These combined studies demonstrate the possibility that CYP1A1 induction could be used to determine the

exposure of and responsiveness to PHAHs and PAHs in marine mammals, including cetaceans.

CYP1A1 and CYP1A2 have overlapping substrate specificities, including the O-dealkylation of 7-methoxyresorufin (7-MR) and 7-ethoxyresorufin (7-ER) (Burke *et al.* 1994). The ethoxyresorufin-O-deethylase (EROD) and methoxyresorufin-O-demethylase (MROD) assays, which measure the metabolism of 7-ER and 7-MR, are commonly employed to measure CYP1A activity. CYP1A1 preferentially catalyzes the hydroxylation and epoxidation of benzo[a]pyrene (Figure 1), which has lead to the use of benzo(a)pyrene monooxygenase (BPMO) activity as an assay of catalytic function. Benzo[a]pyrene epoxide metabolites can react with DNA to form adducts that can lead to point mutations and have been correlated to the development of cancer (Chang *et al.* 1994). Thus, CYP1A induction may not be simply a marker for exposure, but may be a biomarker of susceptibility, as it can have a direct role in toxicity through generation of reactive metabolites. Current research using CYP1A knockout (CYP1A1 $^{-/-}$) mice and zebrafish exposed to CYP1A morphilinos (i.e., knockdown), that functionally remove CYP1A activity, will help to elucidate the potential fundamental role for this protein in PAH and PHAH induced toxicity.

Induction of CYP1A is due to rapidly increased transcription of the CYP1A genes after exposure to inducer compounds. The pathway of induction is well characterized and known to involve several key proteins constitutively expressed in many cells (reviewed in Hahn 1998; Whitlock 1999). The aryl hydrocarbon receptor (AHR) is a cytosolic receptor that binds inducer compounds, such as TCDD (Figure 1). The binding of ligands to the AHR causes the movement of the receptor-ligand product to the nucleus and the release of proteins that interact with AHR in its inactive form (hsp90, Ara9, and p23). Once in the nucleus, AHR binds to a dimerization partner, aryl hydrocarbon receptor nuclear translocator (ARNT). The complex of ligand-AHR-ARNT is competent to bind specific sequences in DNA known as xenobiotic response elements (XREs) or dioxin response elements (DREs). DREs act as an enhancer to increase transcription of AHR responsive genes. This enhancer region appears to interact with a promoter region, which includes a TATA box, to initiate transcription. The increase in transcription causes

an increase in mRNA, CYP1A protein and the metabolism of CYP1A substrates. Thus, induction can be measured by assays of these products including RT-PCR or northern blot for the accumulation of mRNA, western blot and immunohistochemistry for the accumulation of protein, and catalytic assays such as EROD for the increased metabolic capability of the tissue. A diagram of the working model of CYP1A induction is shown in Figure 2.

Both AHR and ARNT belong to a specific group of transcription factors known as bHLH/PAS proteins (basic helix-loop-helix/Per-ARNT-Sim). These proteins seem to be involved in fundamental processes governing homeostasis, circadian rhythms and development. At the N terminus are the basic and helix-loop-helix regions, which are important for DNA binding and protein-protein dimerization, respectively. C terminal to this is the PAS domain, which contains A and B domains enriched with nonpolar residues that are important for dimerization, DNA recognition and ligand binding. The C terminal region contains the transactivation domains of the protein. ARNT is a dimerization partner for many bHLH/PAS proteins and therefore appears to function in multiple signaling pathways. The AHR has been characterized in a bottlenose dolphin kidney cell line (Carvan *et al.* 1995), in the beluga whale (Hahn *et al.* 1992; Hahn *et al.* 1994; Jensen *et al.* 2001) and in the white-sided dolphin (Hahn 1998).

CYP1A enzymes are broadly expressed in vertebrates. In mammals, CYP1A2 and/or CYP1A1 is typically expressed in at least the liver, kidney, lung, gastrointestinal tract and skin. CYP1A enzymes have been found in hepatic parenchyma in all species examined (Baron *et al.* 1981; Dees *et al.* 1982; Foster *et al.* 1986; Rosenberg 1991; Matsuda *et al.* 1995; Dey *et al.* 1999). The expression of CYP1A proteins in mammalian liver is typically zonal; highest expression is centrilobular and becomes panlobular with high induction (Oinonen and Lindros 1998; Drahushuk *et al.* 1999). The same zonal pattern of CYP1A1 expression has been seen in pilot whale liver (Stegeman *et al.* 1994). Regardless of the organ, CYP1A proteins are typically expressed in endothelia (Dey *et al.* 1999). In fish, endothelial CYP1A expression has been seen wherever there are blood vessels including in liver, heart, gill, kidney, gut, spleen, red muscle, brain, and swimbladder rete mirabile (Miller *et al.* 1989; Stegeman *et al.* 1989; Smolowitz *et al.*

1991; Husoy *et al.* 1994; Schlezinger and Stegeman 2000). Thus, CYP1A proteins are widely expressed in vertebrates and have similar patterns of expression across mammalian, and sometimes vertebrate taxa.

Role of CYP1A1 in Toxicity

The induction of CYP1A enzymes is one of the most sensitive responses to the activation of the AHR (Birnbaum and Tuomisto 2000). Most toxicities of PHAHs and PAHs are AHR-dependent (Gonzalez and Fernandez-Salguero 1998), yet it remains unclear whether CYP1A1 induction is required for these toxicities, since the AHR activates many other genes. A direct role of CYP1A1 in PAH and PHAH toxicity has been demonstrated in PAH associated tumors (reviewed in Birnbaum *et al.* 2000) and in the generation of reactive oxygen species (Dalton *et al.* 2002). Other toxicities have been correlated to CYP1A1 induction without a mechanistic link having been established. However, CYP1A1 induction has not been correlated with all toxicities, or in all species. The rank order for the sensitivity of species to CYP1A1 induction and other toxicities is often consistent, such that determining the relative sensitivity of species to CYP1A induction may be useful to infer the relative sensitivity to some PAH and PHAH toxicities.

The generation of reactive oxygen species (ROS) can be cytotoxic to cells, causing damage to DNA, proteins, and lipids (Kappus 1987). Oxidative damage has been found in aquatic species exposed to environmental contaminants (reviewed in Livingstone 2001), including those that activate the AHR. Oxidative stress can be induced by the activation of the AHR through activation of genes involved in inflammation, altering estrogen metabolism, induction of the xanthine oxidase/xanthine dehydrogenase system, and induction of CYP1 family enzymes (reviewed in Dalton *et al.* 2002). The binding of certain substrates in the active site of CYP1A enzymes is thought to generate ROS in several vertebrate species including mammals and fish, due to uncoupling of microsomal electron transfer (Schlezinger *et al.* 1999; Schlezinger *et al.* 2000). This occurs with compounds such as 3,3′,4,4′-tetrachlorobiphenyl and 3,3′,4,4′,5-pentachlorobiphenyl that are poorly metabolized by CYP1A enzymes (Schlezinger *et al.*

1999; Schlezinger and Stegeman 2001). Thus CYP1A enzymes can be mechanistically linked to oxidative stress in cells and directly implicated in diseases associated with oxidative stress.

Induction and polymorphisms of CYP1A enzymes have been linked to some specific cancers in humans, including cancer of the lung, bladder, and breast (Murray *et al.* 1995; Autrup 2000; Firozi *et al.* 2002). A CYP1A polymorphism in Atlantic tomcod has been associated with higher susceptibility to hepatic neoplasms (Roy *et al.* 1995). Members of the CYP1 family metabolize PAHs into reactive metabolites that can covalently bind both DNA and macromolecules in the cell. The presence of PAH-DNA adducts, such as those caused by benzo[a]pyrene, can cause point mutations (Kozac *et al.* 2000), which are known to cause tumors. Thus, mechanistic links for the involvement of CYP1A in PAH related tumors have been established. CYP1A induction has been correlated to bladder tumor grade in humans (Murray *et al.* 1995). In environmentally exposed fish, non-neoplastic lesions have been correlated with bilary PAH metabolites, DNA adducts, and CYP1A induction (Myers *et al.* 1998). However, CYP1B1 and not CYP1A1 or CYP1A2 has been directly linked to the generation of DNA adducts of 7,12-dimethylbenz[a]anthracene (DMBA) and development of ovarian tumors in mice (Buters *et al.* 2003). Thus, for some PAHs, CYP1A enzymes are not directly involved in the generation of metabolites directly linked to tumors.

The direct role of CYP1A enzymes in other toxicities is unknown, yet in several cases, correlations between CYP1A and PHAH or PAH induced toxicities have been determined. The relative sensitivity of avian hepatocyte cultures to CYP1A induction was similar to the relative sensitivity of the species to *in ovo* lethality (Kennedy *et al.* 1996), suggesting a strong correlation between CYP1A induction and *in ovo* lethality in bird species. In fish, early life stage mortality in lake trout was well correlated with CYP1A induction in vascular endothelium (Guiney *et al.* 1997). Lethality in rodent species is not as well correlated with CYP1A1 induction. The relative sensitivity between C57BL/6J (TCDD sensitive) and DBA/2J (TCDD resistant) mouse strains was very similar for both CYP1A1 induction and lethality (Weber *et al.* 1995) but this similarity was not found between the Han/Wistar (TCDD resistant) and Long-Evans

(TCDD sensitive) rat strains (Simanainen *et al.* 2002). The LD50 for guinea pig and hamster differ by ≥ 1000 fold, yet they vary by only 69 fold in the ED50 for induction of EROD activity (Fletcher *et al.* 2001). However, the rank order of sensitivity for CYP1A1 induction and lethality is similar in many rodent species (Safe 1990; Fletcher *et al.* 2001; Simanainen *et al.* 2002).

CYP1A induction has been compared to other acute toxicities such as body weight loss, thymic atrophy, and liver enlargement (Fletcher *et al.* 2001). Guinea pig was the most sensitive for all measures and the rank order of sensitivity between species was the similar for EROD activity and body weight gain but not for liver and thymus weights (Fletcher *et al.* 2001). Comparisons of CYP1A induction and body weight loss are very similar between guinea pig and rat in several studies (Safe 1990; Fletcher *et al.* 2001). In rodent species, the rank order of sensitivity to CYP1A1 induction appears to predict the rank order of sensitivity for some, but not all, PHAH induced acute toxicities.

Evidence suggests that CYP1A may be involved in species differences in arachidonic acid metabolism (Lee *et al.* 1998) and vitamin A depletion (Fletcher *et al.* 2001), both of which are involved in TCDD associated toxicity. Immunotoxicity induced by PHAHs and PAHs occurs at concentrations which induces CYP1A in both fish and rodents (Narasimhan *et al.* 1994; Carlson *et al.* 2002a; Carlson *et al.* 2002b). A direct role for CYP1A1 in some aspects of immunotoxicity, via metabolism of PAHs, has been suggested (Carlson *et al.* 2002a). However, lymphoproliferation was apparent at doses below which CYP1A was induced in medaka (Carlson *et al.* 2002a) suggesting that only certain immunotoxic effects may require CYP1A induction. DMBA associated immunotoxicity has been linked to CYP1B1, and not CYP1A1, expression (Mann *et al.* 1999). CYP1A enzymes are not involved other toxic effects of DMBA, including tumors (Buters *et al.* 2003) and ovarian failure (Matikainen *et al.* 2001). It is unlikely, given that the activation of the AHR induces many genes, that CYP1A1 has a direct role in all PAH and PHAH toxicities. Yet CYP1A1 induction indicates that the AHR pathway has been activated, which is required for PAH and PHAH toxicities. The direct role of CYP1A1 in oxidative stress, tumors, and possibly aspects of immunotoxicity, suggest that measures of CYP1A1 are relevant for studies of PAH and PHAH toxicity.

CYP19 in Mammals

CYP19 encodes the protein aromatase (P450arom), the enzyme responsible for the aromatization of androgens to estrogens (Simpson *et al.* 1994; Simpson *et al.* 2002). Aromatase hydroxylates androgens in the C19 position to convert testosterone, androstenedione and 17-αhydroxyandrostenedione to estradiol, estrone and estriol, respectively (Corbin *et al.* 1988) (Figure 3). In all mammals examined, except the pig, aromatase is encoded by a single gene but in fish, two CYP19 genes (CYP19a and CYP19b) encode for brain and ovary specific aromatases (Tchoudakova and Callard 1998). The full-length aromatase gene has been sequenced from 11 mammalian species (human, macaque, marmoset, pig, horse, cow, goat, sheep, rat, mouse, and rabbit). The human CYP19 gene is extremely large, spanning over 75 kb with at least 16 exons (Faustini-Fustini *et al.* 1999). Interestingly, multiple genes encoding for aromatase have been found in pig but not in other mammalian species (Choi *et al.* 1997). The pig aromatase proteins have distinct catalytic capabilities, as they differ in affinity and metabolic capacity for testosterone but not androstendione (Corbin *et al.* 1999). In pig, different aromatase genes are preferentially expressed in different tissues (Choi *et al.* 1997). In humans and the other mammals, tissue specific expression seems to be controlled by tissue specific promoters for a single gene (Faustini-Fustini *et al.* 1999). Similar to the wide expression of CYP1A, CYP19 is expressed in a number of tissues including ovary, placenta, testis, adipose, muscle, liver, various sites in the brain, hair follicles, genital skin fibroblast and in fetal tissue in humans (Faustini-Fustini *et al.* 1999). However, in the other vertebrates, aromatase appears more restricted in its expression, to gonads and brain and additionally in the placenta in primates and ungulates (Simpson *et al.* 1994; Conley and Hinshelwood 2001; Simpson *et al.* 2002).

The importance of estrogen in mammalian development and reproduction has been investigated by the generation of knockout (CYP19 $^{-/-}$) mice and by studying humans with mutations in CYP19. The dearth of human subjects with mutations in either the estrogen receptor (ER) or CYP19 gene were long thought to point to estrogen's critical role in survival. The generation of an ERα-minus (ERKO) mouse (Lubahn *et al.*

1993) and the identification of mutations in both ERα and CYP19 in humans has proved otherwise.

Recently, CYP19-deficient (ArKO) mice have been generated by disrupting exon 9 (Fisher *et al.* 1998) and exons 1 and 2 (Honda *et al.* 1998). Phenotypically, the ArKO mice are normal at birth (Fisher *et al.* 1998; Honda *et al.* 1998) but development of reproductive organs was abnormal on both a gross morphological and histological basis (Fisher *et al.* 1998; Honda *et al.* 1998). Internal fat stores and gonadal and mammary weights are all increased (Fisher *et al.* 1998; Honda *et al.* 1998; Jones *et al.* 2001) and bone formation and growth are altered in the ArKO mice (Oz *et al.* 2001). FSH and LH are elevated in ArKO mice, suggesting that estradiol is involved in negative feedback regulation of gonadotropins in both male and female mice (Fisher *et al.* 1998). Initially, spermatogenesis appears normal (Fisher *et al.* 1998; Honda *et al.* 1998) however, spermatogenesis, sexual behavior and fertility are eventually disrupted (Fisher *et al.* 1998; Honda *et al.* 1998; Robertson *et al.* 1999). Thus, it appears that aromatase is important for development, bone formation, fat accumulation, hormone regulation, and spermatogenesis and suggests a direct role for estradiol in both female and male fertility.

The fish CYP19a upstream region contains XRE promoter sequences (Callard *et al.* 2001) that would suggest that ovarian aromatase may be induced by PHAHs, similar to CYP1A. Unlike all vertebrate CYP1As, and possibly fish CYP19a, which are induced by PHAHs, mammalian CYP19 activity may be decreased by PHAHs (Drenth *et al.* 1998; Hany *et al.* 1999; Gerstenberger *et al.* 2000). The evidence for PHAH suppression of CYP19 is limited and the mechanism of suppression is unknown. In rats exposed prenatally to a reconstituted PCB mixture that included several mono-*ortho* (CB28 and 105) and non-*ortho* PCBs (CB77, 126, and 169), aromatase activity was decreased in the hypothalamic/preoptic area of the brain in newborn males (Hany *et al.* 1999). Ovarian aromatase activity was not affected in female offspring in that study, although a significantly lower estradiol/testosterone ratio was seen in the dams (Hany *et al.* 1999). Rats exposed *in utero* to PCB, organochlorine and mercury contaminated fish collected from the Great Lakes showed decreased ovarian aromatase activity without a concomitant decrease in estradiol (Gerstenberger *et al.* 2000). The congener specific PCB profile from

that study is unknown but total PCB consumption was an estimated 14 mg over the course of the experiment. TCDD, CB 126 (a planar PCB that induces CYP1A1) and benzo[a]pyrene decreased aromatase activity in human placental JEG-3 cells in culture (Drenth *et al.* 1998). The effect of TCDD and CB 126 on aromatase was suggested to be AHR dependent, since Aroclor 1016, an industrial PCB mixture that contains a preponderance of lesser chlorinated PCBs having a low affinity for the AHR, did not affect aromatase activity (Drenth *et al.* 1998). However, the decrease in aromatase activity in JEG-3 and JAR choriocarcinoma cells exposed to a variety of organochlorines may have been related to cytotoxicity (Letcher *et al.* 1999). Further research is needed, not only to address the effects of PHAHs on aromatase activity, but also whether the mechanism is AHR-dependent and if it occurs via a direct interaction of compounds on the enzyme or by alteration of expression.

Specific Objectives

While studies support a link between contaminant exposure and toxicity in certain marine mammal populations, there have been few approaches that determine the biological responses of marine mammals to environmental contaminants. While the measurement of contaminant concentrations is important, without information on the responsiveness or sensitivity of the species to the specific contaminants measured, we cannot infer whether toxicity is likely. CYP1A1 expression is known to increase after exposure to PHAHs and CYP1A1induction has been correlated to toxicity in rodents. The measurement of CYP1A1 in cetacean species, and the correlation of CYP1A1 expression to contaminant concentrations will allow us to assess which cetacean species are most at risk of contaminant toxicity. I have examined the relationship between CYP1A1 and contaminants in three species: the beluga whale (*Delphinapterus leucas*), the white-sided dolphin (*Lagenorhynchus acutus*) and the bottlenose dolphin (*Tursiops truncatus*). Internal organs from beluga whale (Chapter 2) sampled during subsistence hunts in the Arctic and from stranded animals in the St. Lawrence Estuary offer a rare chance to examine populations with both high (St. Lawrence Estuary) and low (Arctic) exposures to PHAH and PAH contaminants. The St. Lawrence Estuary beluga have very

high contaminant concentrations in blubber and suffer from unusual lesions and tumors that are not seen in other cetacean populations. Internal organs from white-sided dolphin (Chapter 3) were collected during mass stranding events. These apparently healthy animals were subject to intense necropsy and represent our most comprehensive set of samples to date. Contaminant concentrations were performed in blubber for comparison with CYP1A1 catalytic activity and expression levels, similar to what had been previously done with beluga. Direct comparisons of CYP1A1 expression in various internal organs can give us a picture of the degree to which CYP1A1 is induced in the beluga and white-sided dolphin by contrasting the pattern and extent of expression with other mammalian species. CYP1A1 expression was determined in integument (skin, skin and blubber) biopsies collected from free ranging bottlenose dolphin (Chapter 4). The coastal population from which these animals were biopsied is well studied and the impacts of age, sex and contaminant concentrations on CYP1A1 expression could be explored in a single study. This research will allow us to assess the necessity of life-history parameters for interpretation of CYP1A1 expression data from free-ranging animals where this information is most often lacking. Last, I have cloned and sequenced CYP19 (aromatase, P450arom) from a white-sided dolphin ovary and performed transient transfections of the full-length cDNA to determine the functional characteristics of dolphin aromatase (Chapter 5). The evolution and functional conservation of aromatase across vertebrate taxa was determined. This research gives us the molecular tools to monitor aromatase expression in cetaceans and assess the possible effects of PHAHs on estrogen production and function.

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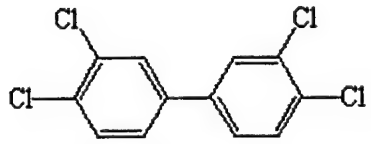
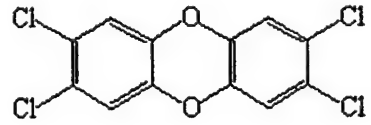
Figure 1. Chemical Structures of Typical PHAHs, PAHs, Pesticides and PBDEs.

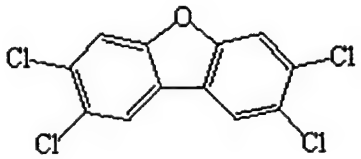
CYP1A1 Inducers



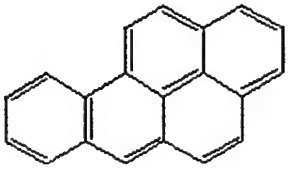
PCB 126
(3,3',4,4'-tetrachlorobiphenyl)



TCDD
(2,3,7,8-tetrachlorodibenzo-*p*-dioxin)

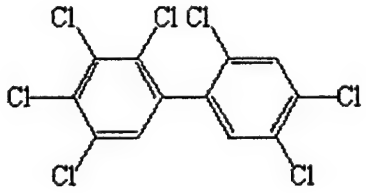


TCDF
(2,3,7,8-tetrachlorodibenzofuran)

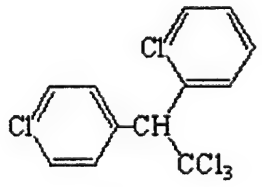


Benzo(a)pyrene

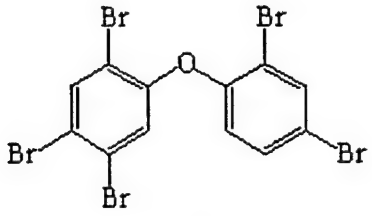
Non-CYP1A1 Inducers



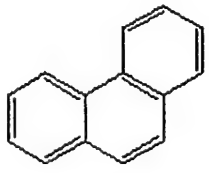
PCB 180
(2,2',3,4,4',5,5'-heptachlorobiphenyl)



DDT
(*o,p'*-dichlorodiphenyltrichloroethane)



PBDE 99
(2,2',4,4',5-pentabromodiphenylether)



Phenanthrene

Figure 2. Model of CYP1A1 induction pathway. Taken from (Whitlock 1999).

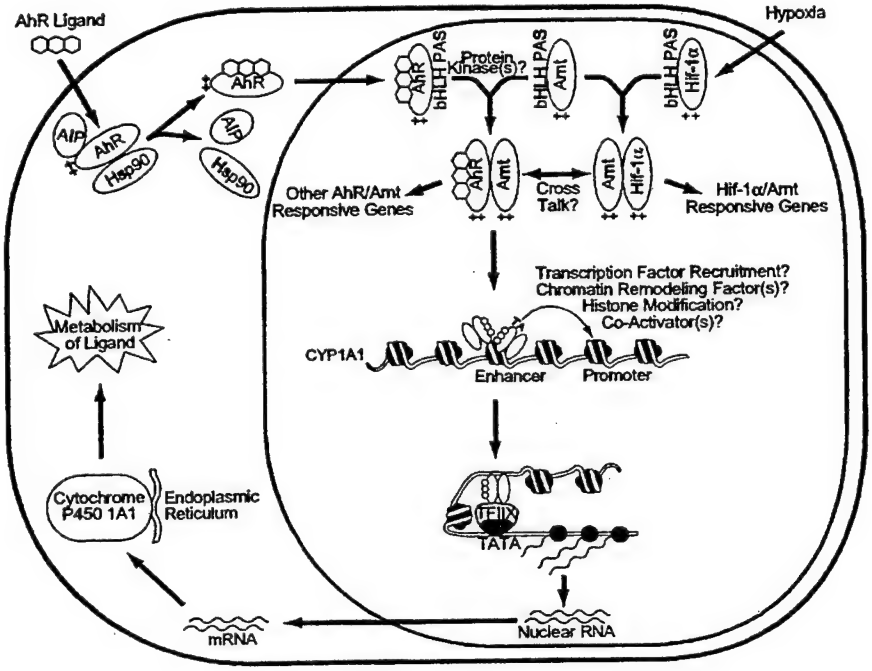
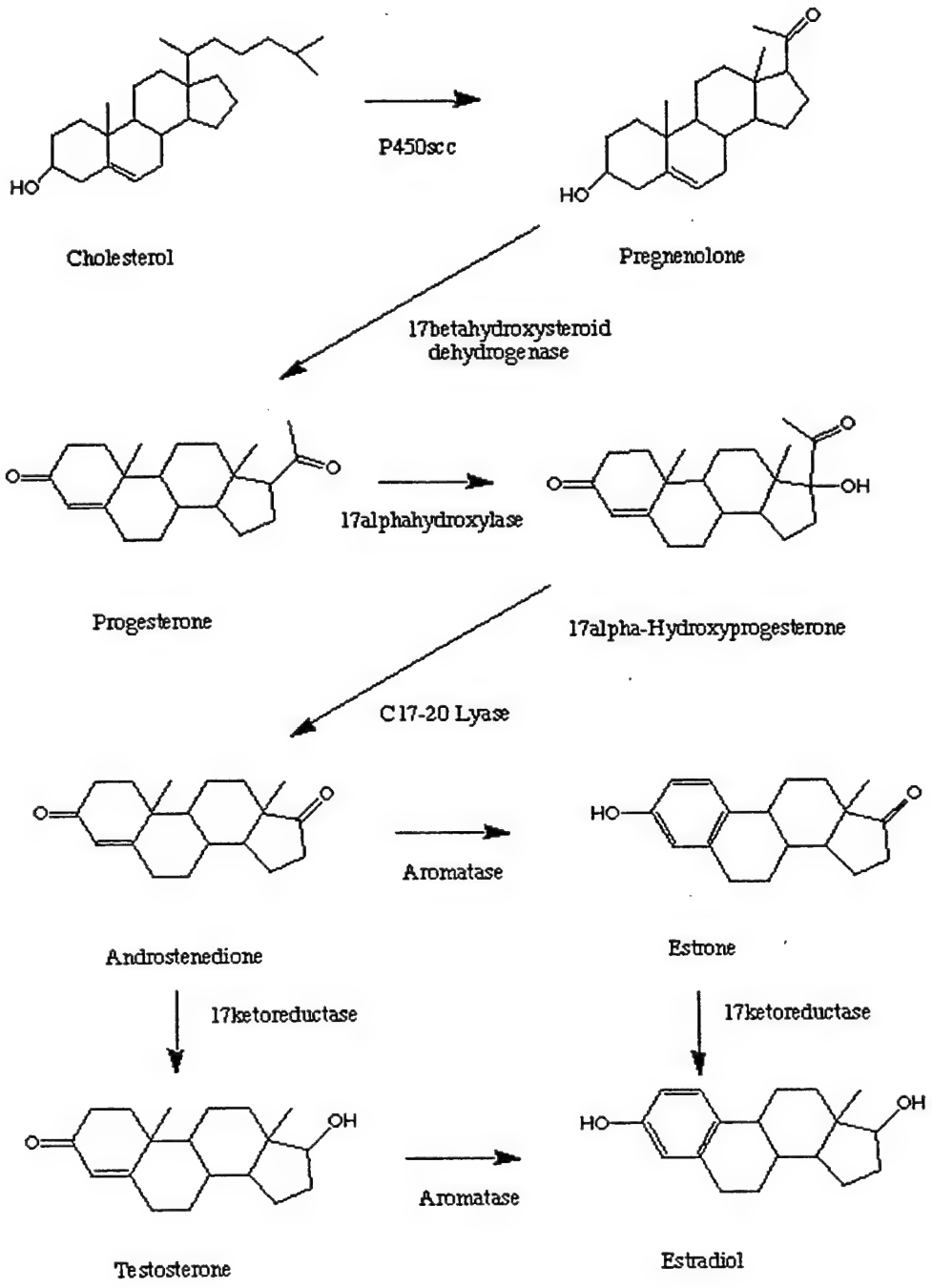


Figure 3. Estradiol synthesis pathway. Only major pathway shown.



Chapter 2. Impact of Arctic Pollutants: CYP1A1 expression in Beluga whales indicates systemic effects

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Abstract

High level CYP1A1 induction has been correlated with toxic effects in mammals [1]. This protein is induced by exposure to polycyclic aromatic hydrocarbons (PAHs) and planar halogenated aromatic hydrocarbons (PHAHs) such as non-*ortho* polychlorinated biphenyls (PCBs) [2]. Immunohistochemistry revealed that cytochrome P450 1A1 (CYP1A1) protein was highly expressed in several internal organs of beluga whale from two locations in the Arctic and from the St. Lawrence estuary. The pattern and extent of CYP1A1 expression in these whales are similar to those seen in animal models that are strongly induced. The strong induction of beluga CYP1A1 even at the lower doses of environmental contaminants in the Arctic suggests that this species is highly sensitive to CYP1A1 inducers. The high level of CYP1A1 expression coupled with high levels of contaminants that include CYP1A1 substrates in St. Lawrence beluga indicate that CYP1A1 could be involved in the development of neoplastic lesions seen in the St. Lawrence beluga whale population [3]. Since beluga have a systemic response to PHAH and PAH contaminants at both high and low doses, toxic effects may be expected in Arctic populations.

Introduction

Chemical contaminants are found throughout the world's oceans. Specific health effects and overt disease in fish have been linked to high concentrations of contaminants in some coastal regions of North America [4,5]. Atmospheric processes distribute many of these same contaminants to the polar regions [6], where they accumulate in the fatty tissue of top predators. Concentrations of contaminants in Arctic ecosystems have declined somewhat over the past 20 years, yet contaminants persist at appreciable levels in marine mammal species including the beluga [7]. While cetaceans in the Arctic have contaminant concentrations that are at least 10 times lower than the most highly contaminated cetaceans [8], these animals may yet be at risk for adverse health effects.

Arctic contaminants fall into 5 broad categories: chlorinated industrial compounds including polychlorinated biphenyls (PCBs), organic pesticides, polycyclic aromatic

hydrocarbons (PAHs), metals, and radionuclides [6]. Of these, persistent contaminants that accumulate in the lipid rich blubber of whales include PCBs, DDT (dichlorodiphenyltrichloroethane) and other chlorinated pesticides. Odontocetes (toothed whales) may be at the greatest risk of contaminant effects because these animals are top predators that accumulate contaminants to a higher degree than do mysticetes (baleen whales) [9]. Concentrations of PCBs as high as 300 $\mu g\ g^{-1}$ (lipid weight basis) have been recorded in odontocete blubber [10]. Planar halogenated aromatic hydrocarbons (PHAHs), the dioxin-like contaminants that include non-*ortho* and mono-*ortho* substituted PCBs, are of special concern because even at low doses they can affect development of the immune, nervous, and reproductive systems in animal models[11].

Assessing health effects of contaminants in cetaceans is difficult because experimental exposures are precluded and fresh tissues are rarely available. Molecular changes associated with exposure to selected compounds can suggest whether systemic effects are likely. Cytochrome P450 1A (CYP1A) induction is elicited by PAHs and PHAHs via binding to the aryl hydrocarbon receptor (AHR)[2]. CYP1A induction has been correlated to higher order toxic effects including thymic atrophy, weight loss and lethal toxicity induced by PCB, PCDD and PCDF exposure in rodents [1]. Thus, systemic CYP1A expression can indicate a risk for toxic effects.

The CYP1A gene subfamily contains two members in mammals: CYP1A1 and CYP1A2. Typically in mammals, CYP1A2 expression is limited to liver while CYP1A1 is more widely expressed in extrahepatic organs as well as liver of induced animals. In non-mammalian vertebrates including fish, the members of this gene subfamily are widely referred to as CYP1A although multiple members do occur in some species.

In an earlier study, CYP1A1 levels detected by western blot in liver microsomes of Arctic beluga were strongly correlated to the concentration of non-*ortho* and mono-*ortho* PCBs in blubber, compounds that are known to induce CYP1A1 through the AHR [12]. In this study we employed immunohistochemistry to examine the cellular location and relative levels of CYP1A1 expression in multiple organs of beluga whale from the St. Lawrence estuary and the Arctic (Beaufort Sea and western Hudson Bay stocks). Native

subsistence hunts for beluga in the Arctic regions make it possible to obtain organs from freshly killed animals, which is not possible for many other cetacean species. Beluga from the St. Lawrence estuary population have high concentrations of PCBs, chlorinated pesticides [13], and metal (mercury, lead, and selenium)[14] contaminants in blubber. These animals show highly elevated prevalences of overt pathologies linked to toxicants [15]. Therefore, we addressed the question of whether CYP1A1 might be expressed in multiple organs of beluga from several regions, as this would indicate whether biochemical effects in these animals might be occurring systemically and could indicate the sensitivity of this species to PHAH toxicity.

Results and Discussion

Immunohistochemical analyses used the monoclonal antibody 1-12-3, which is highly specific for CYP1A1 in mammals [16] and recognizes a single microsomal protein band in beluga liver [12]. Analysis of liver and extrahepatic organs showed patterns of expression consistent with a strong induction of CYP1A1, based on comparison with induced mammalian and non-mammalian vertebrate models (Tables 1, 2 and supplementary data). CYP1A1 expression was seen in vascular endothelial cells in all organs examined in most individuals included in this study (Table 2). The widespread expression of endothelial CYP1A1 is the first biological indication of a systemic effect of contaminants in the Arctic beluga whale.

CYP1A1 was highly expressed in hepatic parenchyma of Arctic beluga (Table 1). Typically in mammalian liver, CYP1A1 expression is localised to periportal parenchyma in untreated or slightly induced animals and pan-lobular expression is seen only in animals in which CYP1A1 is strongly induced [17]. High level CYP1A1 expression that is pan-lobular (Figure 1) is fully consistent with strongly induced CYP1A1 in liver of Arctic beluga. Surprisingly, CYP1A1 expression in liver from the highly contaminated St. Lawrence beluga was significantly lower (Table 1) in spite of a greater exposure to inducing compounds: liver PCB concentrations in male beluga average 1445 ng g^{-1} and 132 ng g^{-1} in the St. Lawrence and Arctic, respectively [18]. CYP1A1 expression may be

suppressed in St. Lawrence beluga liver, potentially as a result of high level contaminant exposure. CYP1A is suppressed in liver but not other organs of fish experimentally exposed to high doses of non-*ortho* PCB congeners (eg. CB126)[19]. The St. Lawrence beluga samples were from stranded animals that die and wash ashore and for which the time of death is unknown. In contrast, the Arctic samples were from hunted animals that were immediately taken to shore for sampling. The time from death to necropsy was longer for the St. Lawrence beluga than for the Arctic beluga and histological analyses do show autolysis in the St. Lawrence beluga samples. Although CYP1A1 expression in other organs was not significantly lower in the St. Lawrence beluga than in Arctic animals, the liver degrades at a faster rate because heat is generated by bacterial fermentation in the gut and liver contains both endogenous and bacterial enzymes, including proteases, that digest the tissue. It is likely that differences in hepatic CYP1A1 expression reflect degradation and the difference between samples collected from subsistence hunts and strandings.

Endothelial CYP1A1 expression (Table 2) was high in lung. CYP1A1 expression was also seen in chondrocytes and bronchiolar epithelium but was not seen in type I or type II pneumocytes (see supplementary data). The predominant exposure for CYP1A1 inducers is dietary, but a recent study in mice suggests PCB uptake can be greater via inhalation than from diet [20]. Thus, consideration of non-dietary exposures such as inhalation may be warranted in regions where high PAH and/or PCB exposures are likely. Interestingly, many Arctic contaminants including PCBs, are derived from atmospheric sources. Atmospheric sources of PCBs could result in an estimated lung exposure of 1.3 – 67 ng day^{-1} in Arctic beluga (see Box 1). Likewise, an inhaled PAH exposure could be expected to range from 5.6 – 363 ng day^{-1} in Arctic beluga (see Box 1) although this exposure would be dominated by lower molecular weight PAHs such as fluorene and phenanthrene [6], which typically do not induce CYP1A [21]. Hormonal, histopathological and behavioural changes were seen in mice exposed to 0.9 μg m^{-3} Aroclor 1242 in the air [20], a concentration which is ~10000 fold lower than Arctic atmospheric PCB concentrations [6]. Considering that type I pneumocytes (the primary cell type involved in

gas exchange in the lung) did not express CYP1A1 in lung, it is more plausible that CYP1A1 induction in lung was solely the result of dietary exposure and that the contribution of inhaled contaminants was marginal.

In bladder, CYP1A1 was highly expressed in both endothelium (Table 2) and transitional epithelium (Table 1). The transitional epithelium forms the bladder mucosa: CYP1A1 expression was highest in umbrella cells, which form the outermost layer of the epithelium and are in direct contact with urine. In humans, CYP1A is involved in the activation of a variety of potential bladder carcinogens [22], is expressed in bladder tumors, and has been correlated to bladder tumour grade [23]. A transitional cell carcinoma of the bladder has been found in an animal from the highly contaminated St. Lawrence beluga [24] and such high CYP1A1 expression in this cell type suggests that CYP1A1 could be involved in the development of bladder tumor in the St. Lawrence beluga population.

The expression of CYP1A1 in bladder was as high in the Arctic as in the St. Lawrence beluga. Considering that in the transitional epithelium, the most highly induced cells were in direct contact with urine (Figure 3), the induction of CYP1A1 in bladder presumably was caused by contaminants in the urine. Potential CYP1A1 inducers in urine include both PCBs and PAHs. PAHs are higher in concentration in the St. Lawrence estuary than in the Arctic: sediment associated PAHs are 500-4500[25] and 400-980 ng g^{-1} [6] in the St. Lawrence and Arctic, respectively. The relative contribution of PCBs and PAHs to urinary contaminants is unknown in the St. Lawrence beluga. In the Arctic ecosystem, where many contaminants are atmospherically derived, atmospheric PAHs are dominated by those that do not induce CYP1A1[6]. The contribution of geochemically derived PAHs to the Arctic food web, and beluga whale specifically, is not clear. Considering that mono-*ortho* PCBs are highly correlated to liver CYP1A1 induction in Arctic beluga [12], PAH exposure is likely low. Thus, for Arctic beluga, only PCB, and not PAH, contributions to urinary contaminants needs to be considered. In mice exposed to PCBs, only 5% of oral PCBs appeared in urine, mainly as conjugates [26]. We can calculate a conservative whole body dose of PCBs in Arctic beluga (see Box 2) to estimate a reasonable upper limit on an oral dose. The urinary PCB concentrations

would be very small in the Arctic beluga, because the daily oral dose would likely be less than the accumulated whole body dose of 1.08 to 1.96 mg kg^{-1}. These results suggest that the doses required for CYP1A1 induction in bladder are low, and again indicate that beluga are very sensitive in their responses to PHAH contaminants.

Moderate levels of CYP1A1 expression were seen in the spermatogenic series in the testis (Table 3). Although this may have included some Sertoli cells, CYP1A1 expression appeared primarily in spermatogonia and spermatocytes. In other mammals, testicular microsomal preparations have shown very low or no CYP1A catalytic activity in multiple mammalian species [27-29]. Testicular CYP1A activity was not induced in rats [28], bulls [27], or mice [29] exposed to a variety of inducers, although the AHR and dimerization partner ARNT, which are required for CYP1A induction, are present in testis [28]. Immunohistochemical analyses of mouse testes showed CYP1A1 activity in interstitial cells only, and this was reported not induced by benzo[a]pyrene[29]. Thus, CYP1A1 expression in the spermatogenic series is an unusual finding in a mammalian species. Considering that CYP1A1 is involved in the activation of pro-carcinogens and generation of reactive oxygen species, high level CYP1A1 expression in the spermatogenic series could be significant for sperm function and gamete development.

The high levels of CYP1A1 expression are consistent with exposure of the beluga whale from both the Arctic and the St. Lawrence estuary to concentrations of environmental contaminants that are highly potent in these animals. Interestingly, the level of response was not markedly different between animals from the St. Lawrence estuary and the Arctic in spite of significant differences in contaminant exposure and apparent tumor prevalence. No tumors where found in 50 Arctic beluga examined, while 21 tumors were found in 100 St. Lawrence beluga, resulting in an annual cancer rate of 163 per 100 000 animals calculated for the St. Lawrence estuary beluga [30]. However, the necropsies on the Arctic beluga were not as detailed as necropsies on the St. Lawrence animals and the Arctic animals examined were much younger. Until detailed necropsies are performed on older Arctic animals, it will be difficult to determine if tumors occur in Arctic populations of beluga. Liver CYP1A1 expression was shown previously to be

highly correlated to mono-*ortho* and non-*ortho* PCBs, ligands for the AHR, in the blubber of Arctic beluga whale [12]. The high levels and cellular patterns of CYP1A1 expression are similar to those seen in animal models exposed to high levels of inducers; other mammals do not show this broad pattern of induction unless exposed to high concentrations of contaminants. Thus, even the concentrations of contaminants seen in the Arctic animals, appear able to highly induce CYP1A1 in beluga.

The sensitivity of beluga to CYP1A1 inducers may be reflected in other beluga proteins. The beluga AHR, a transcription factor that largely governs CYP1A1 induction, has been cloned, expressed and shown to bind 2,3,7,8-tetrachlorodibenzo-p-dioxin (a prototypical inducer) with a similar binding affinity to that of the C57BL/6 strain mouse [31]. This strain of mouse is highly sensitive to PHAH toxicity [32], therefore beluga may be similarly sensitive to these contaminants.

In the St. Lawrence estuary only beluga, and not other resident cetacean species, have been found with tumors [33] indicating that beluga may be particularly susceptible to carcinogenic contaminants. The fact that beluga show a systemic response to CYP1A1 inducers, even at lower doses, indicates that other toxic effects elicited by AHR agonists may be expected, even in populations from the relatively uncontaminated Arctic.

Methods

Internal organs were sampled from dead beluga that stranded along the St. Lawrence estuary and during subsistence hunts in the Mackenzie Delta and Hudson Bay. The Mackenzie Delta and Hudson Bay sites represent two separate Arctic populations of beluga, both of which have significantly lower levels of contaminants than the St. Lawrence estuary site (Table 3). The 41 animals included in this study ranged in age from 0-31.5 year (Table 3). Internal organs sampled included adrenal, brain, bladder, colon, gonad (ovary or testis), heart, kidney, liver, lung, skin and thyroid. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 5μm. CYP1A1 expression was determined by immunohistochemistry with the monoclonal antibody 1-12-3, as previously described [34]. This antibody recognises an epitope specific

to CYP1A1 in mammals and detects CYP1A in taxonomically diverse vertebrates including cetaceans [16]. A semi-quantitative index (0-15) of CYP1A1 expression was calculated by multiplying the intensity (0-5) and occurrence (0-3) of label for each cell type in a given organ. A linear relationship between this index and CYP1A protein content measured by immunoblot was previously shown for liver [35].

Acknowledgments

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Table 1. CYP1A1 Expression in Selected Internal Organs of Beluga Whale.

Site		Liver Hepatic Parenchyma	Bladder Transitional Epithelium	Testis Spermatogenic Series[3]
Mackenzie Delta	M	11.7[1]	8	6.3
	F	10	-[2]	-
Hudson Bay	M	12.4	-	3
	F	10	12	-
St. Lawrence	M	0.3*	6	4.5
	F	3.3*	11.25	-

[1]CYP1A1 expression shown are mean scores and on a scale of 0-15. [2]- organ was not available. [3]may include some Sertoli cells. *Mean is significantly different than other sites at $p<0.05$.

Table 2. Endothelial CYP1A1 Expression in Internal Organs.

Site		Brain	Bladder	Gonad	Kidney	Liver	Lung
Mackenzie	M	1.6[1]	8	5	6.5*	3.75	9.1
Delta	F	1.5	-[2]	2.25	9*	2	8
Hudson Bay	M	1.7	-	1	1.8	2.8	6.7
	F	0	0	-	0	0.7	5.5
St. Lawrence	M	2.3	7.6	2.7	0	0	7
	F	3	13.1	1.25	2.2	1.6	6.3

[1]CYP1A1 expression shown are mean scores and on a scale of 0-15. [2]- organ was not available. *Mean is significantly different than other sites at $p<0.05$.

Table 3. Sample Summary.

Site	Number	Age (years)	PCB Concentrations[1] ($\mu g\ g^{-1}$)
Mackenzie	12 Male	> 9[2]	4.9
Delta	3 Female	$4 - 5$[2]	-
Hudson Bay	9 Male	4.5 - 13	2.7
	3 Female	7 - 17.5	-[3]
St. Lawrence	7 Male	0 - 26	78.9
	7 Female	5 - 31.5	29.6

[1] PCB concentrations were determined in blubber and are based on published data [13] and not from samples included in this study. [2] Based on age-length relationships [36]. [3]- data not available.

Box 1. PCB and PAH Dose to Lungs of Arctic Beluga Whale via Inhalation

Volume Inspired is 0.29 - 7.1×10^5 L day^{-1}

Tidal volume (L) × respiration rate (breaths min^{-1}) × 1440 min day^{-1}

Tidal volume (L):

10 – Bottlenose dolphin[1]

62 – Grey Whale[1]

Respiration Rate (breaths min^{-1}):

2 – Bottlenose dolphin[2]

8 – Weddell seal[1]

Lung Dose is 1.3 – 67 ng day^{-1} PCBs and 5.6 – 363 ng day^{-1} PAHs

Air concentration (pg m^{-3}) × Volume Inspired (L day^{-1}) × 0.001m^3 L^{-1}

Air concentrations (pg m^{-3})[3]

PCBs: 44 – 94.3

PAHs: 194 - 508

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Box 2: Conservative Whole Body Burden of PCBs in Arctic Beluga Whale

Body Weight in Arctic Beluga in this Study: 474 – 995 kg

Length: 335 – 447 cm, (*Weight (kg)= $10^{-3.84}$ Length(cm) $^{2.58}$*)[1]

Blubber Weight: 189.6 – 398 kg

Body weight (kg) × % body weight as blubber

Blubber weight is 40% body weight in beluga whale

Whole Body Burden: 1.08 – 1.96 mg kg^{-1}

Blubber contaminants (mg kg^{-1}) × Blubber Weight (kg) ÷ Body weight (kg)

Blubber PCB Contaminants[3]: 2.7 – 4.9 μg g^{-1}

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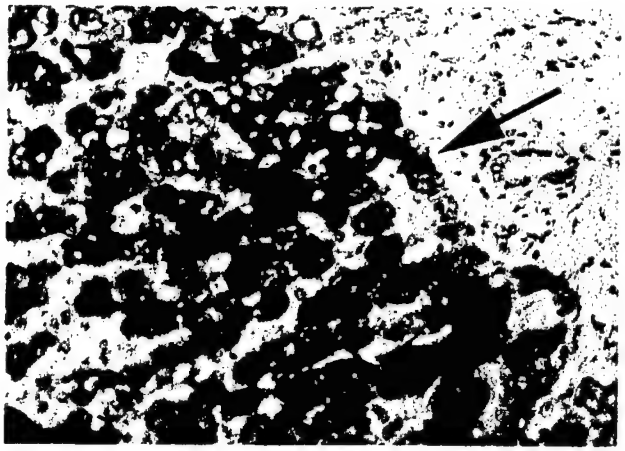
Figure 1. CYP1A1 expression in liver from beluga whale. All images under 400x magnification. CYP1A1 is labeled pink to dark red in color, arrows indicate cells with labeling, arrowheads show the identical cell type without labeling. A) CYP1A1 expression in hepatic parenchyma from an Arctic beluga, B) Serial section from Arctic beluga shown in panel A stained using a non-specific antibody, C) CYP1A1 expression in hepatic parenchyma from a St. Lawrence beluga.



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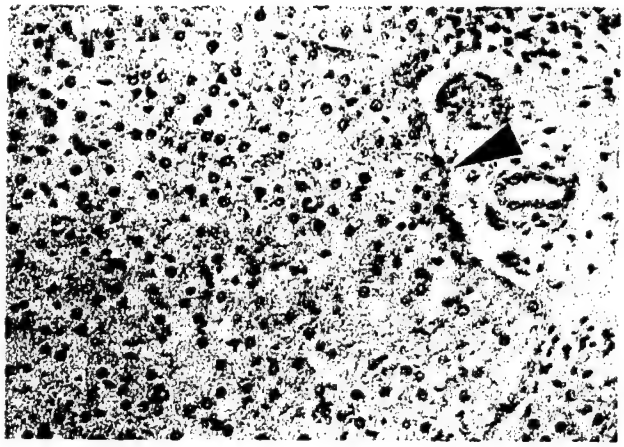

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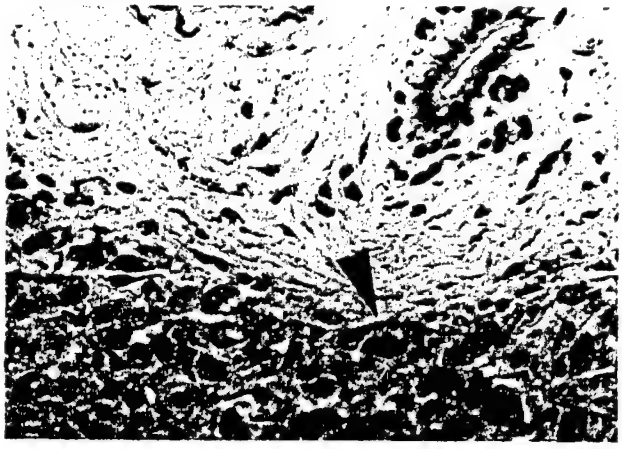
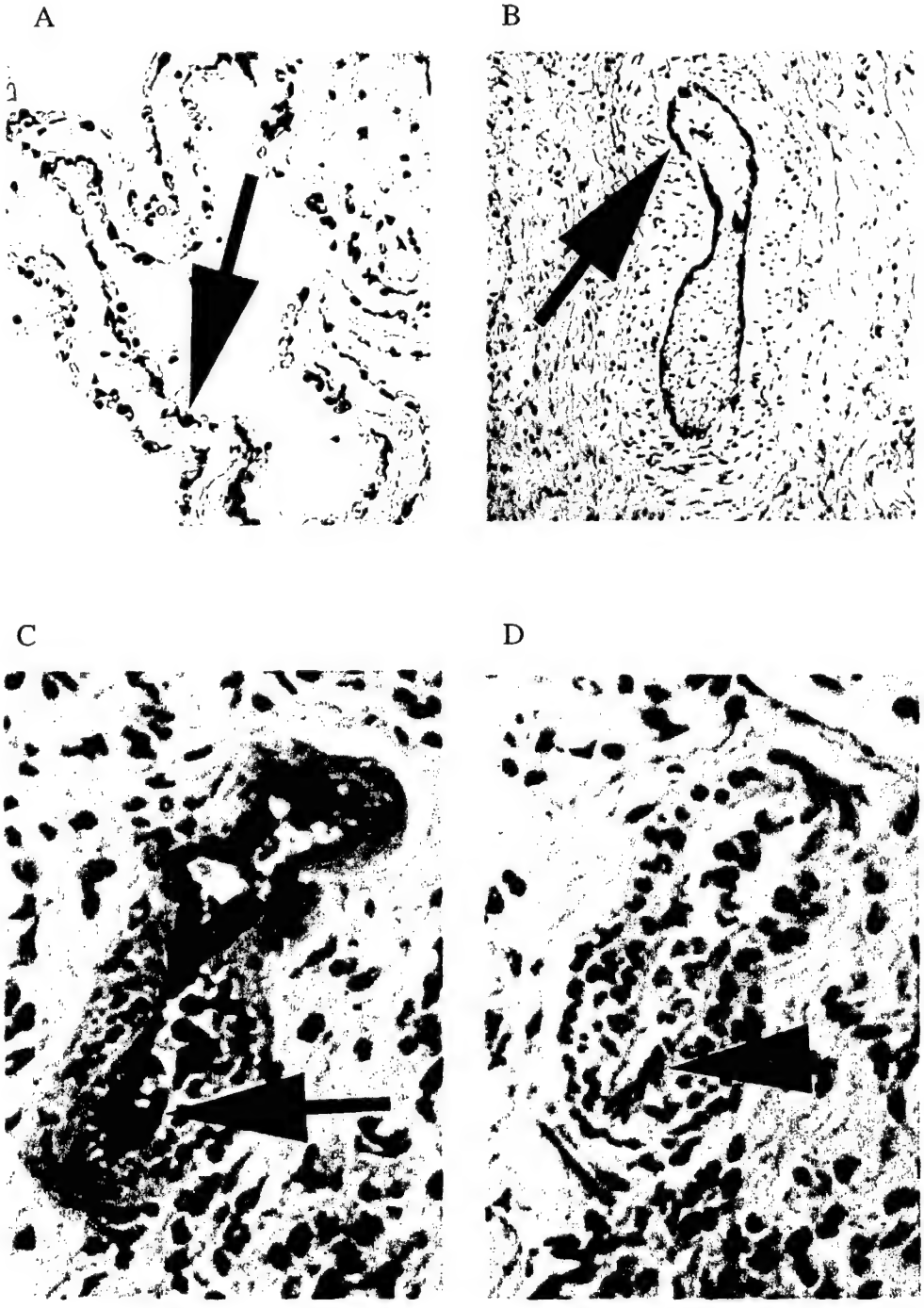


C

Figure 2. CYP1A1 expression in lung and bladder endothelium from beluga whale. All images under 400x magnification except panel B, which is under 200x magnification. CYP1A1 is labeled pink to dark red in color, arrows indicate cells with labeling, arrowheads show the identical cell type without labeling. A) CYP1A1 expression in lung endothelium from an Arctic beluga, B) CYP1A1 expression in endothelium from an arteriole from bladder from an Arctic beluga, C) CYP1A1 expression in endothelium from an arteriole from lung from an Arctic beluga, D) Serial section from Arctic beluga shown in panel C labeled using a non-specific antibody.


A
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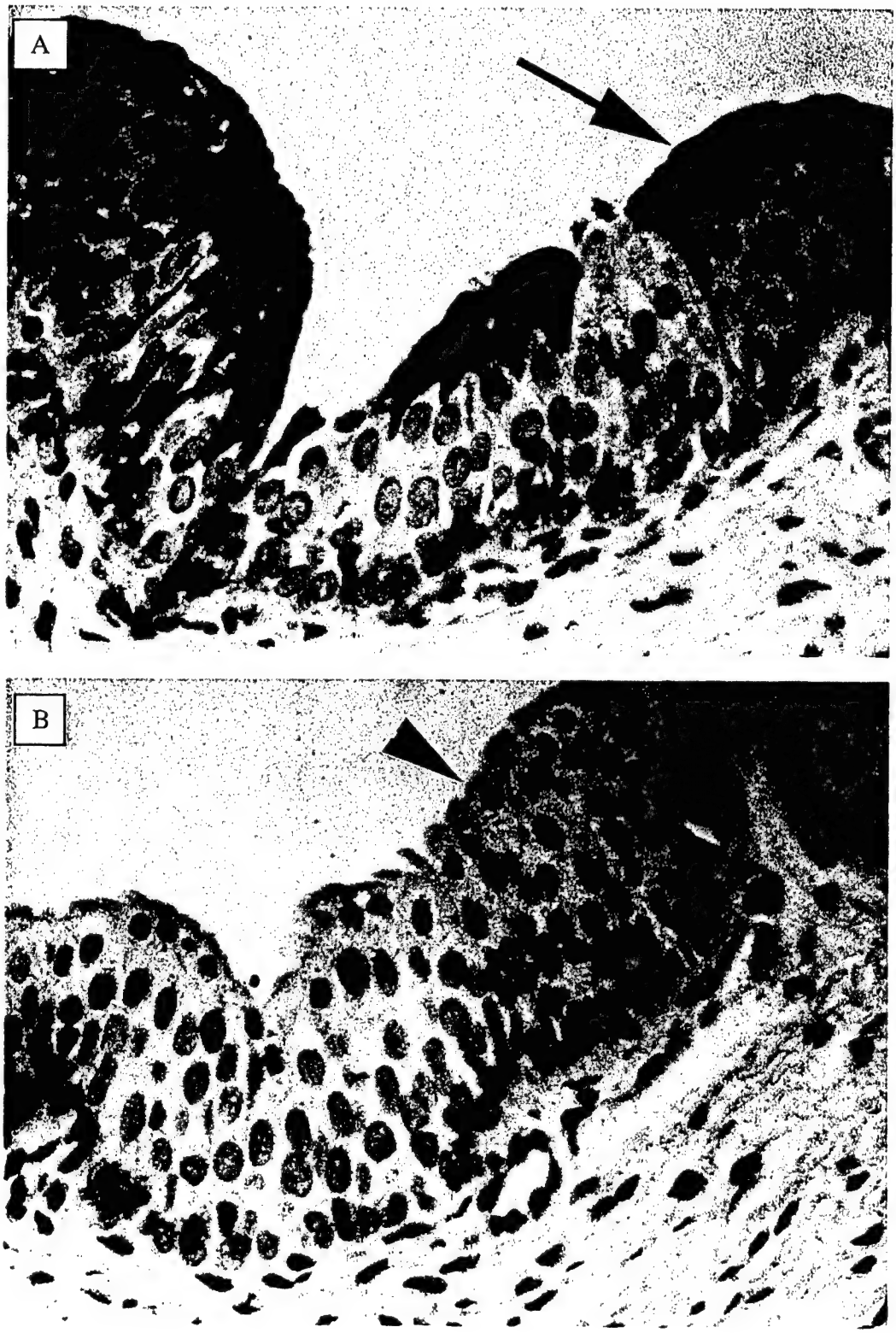
D

Figure 3. CYP1A1 expression in transitional epithelium from the bladder of Arctic beluga whale. All images under 400x magnification. CYP1A1 is labeled pink to dark red in color, arrows indicate cells with labeling, arrowheads show the identical cell type without labeling. A) CYP1A1 expression in the transitional epithelium of bladder, labeling is most intense in the umbrella cells; B) Serial section from beluga shown in panel A labeled using a non-specific antibody.


A
B

Chapter 3. Cytochrome P450 1A1 (CYP1A1) Expression in White-sided Dolphin (*Lagenorhynchus acutus*) Stranded on Cape Cod, MA

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Abstract

Estimating the risk of toxic effects of environmental contaminants in marine mammals will require measurement of early biochemical and molecular responses and their relationship to contaminant concentrations. We have characterized microsomal systems and measured the expression and catalytic activity of cytochrome P450 1A1 (CYP1A1) in multiple internal organs of male and female white-sided dolphin (*Lagenorhynchus acutus*) from the northwest Atlantic Ocean. The total P450 content, cytochrome b5 content, and NADPH-cytochrome c (P450) reductase activity averaged 0.03 nmol mg^{-1}, 0.12 nmol mg^{-1}, and 206.7 nmol mg^{-1} min^{-1} in liver microsomes, respectively. NADPH-cytochrome c (P450) reductase activity was lower in lung (119.4 nmol mg min^{-1}) and kidney (124.9 nmol mg^{-1} min^{-1}) than in liver but was still higher than that reported in liver of most other cetacean species. Catalytic activity of CYP1A1, as measured by ethoxyresorufin-O-deethylase (EROD) activity, was low in all tissues other than liver. In liver, EROD activity ranged from 9.2 – 376 pmoles mg^{-1} min^{-1}. CYP1A1 content was low in all organs, less than 3 pmoles mg^{-1}. EROD activity and CYP1A1 content did not correlate, even when the analysis was limited to liver and not other organs. The lack of correlation between EROD activity and CYP1A1 content was most likely caused by such low levels of CYP1A1 and the variability introduced by measuring such small amounts of protein. Hepatic EROD activity, but not CYP1A1 content, was well correlated with total PCB and Σmono-*ortho* PCB concentrations in blubber. Length, as a proxy for age, did not correlate with EROD activity or CYP1A1 expression. Sex did not appear to influence the relationship of CYP1A1 to contaminant concentrations. Therefore, contaminant concentrations, but not life-history parameters such as age and sex, appear to be determinants of CYP1A1 levels in white-sided dolphins. While catalytic function of CYP1A1 was correlated to Σmono-*ortho* PCBs in white-sided dolphin, CYP1A1 content was correlated to Σmono-*ortho* PCB concentrations in beluga whale (White *et al.* 1994). Based on comparisons of contaminant concentrations and measures of CYP1A1, white-sided dolphins appear to be less sensitive to the induction of CYP1A1 by PCBs than beluga whale. Similar comparison with several species of

cetaceans from the northwest Atlantic Ocean, that share similar habitat and feeding ecology, indicate that it is unlikely that white-sided dolphin are the species most sensitive to PCBs in this region.

Introduction

Anecdotal and epidemiological studies of marine mammal populations have suggested that exposure to high levels of contaminants is immunotoxic, carcinogenic and toxic to the reproductive system. High organochlorine contaminant concentrations, usually including polychlorinated biphenyls (PCBs), have been related to mortality associated with viral and/or bacterial infections in seals (Ross *et al.* 1996), bottlenose dolphin (Lipscomb *et al.* 1994; Lahvis *et al.* 1995; Schulman *et al.* 1997), striped dolphin (Guitart *et al.* 1996), common dolphin (Birkun *et al.* 1999), and harbour porpoise (Jepson *et al.* 1999). The highly contaminated beluga whales from the St. Lawrence Estuary suffer from an unusually high tumor prevalence (Martineau *et al.* 1994). Reproductive abnormalities have been found in seals with exposure to increased contaminant concentrations (Reijnders 1986). These data suggest a causal relationship between contaminants and toxic impacts in marine mammal populations.

The odontocetes, or toothed whales and dolphins, typically have higher contaminant concentrations than the mysticetes or baleen whales (O'Shea and Brownell 1994). Because contaminants are from anthropogenic sources, we expect coastal populations of odontocetes may have the highest exposures and risk of toxicity. Although much effort has been made to determine which populations have high contaminant exposure, biological data, particularly data indicating the responsiveness to contaminants, are greatly needed to identify those species most sensitive to contaminant exposure. Measuring early molecular or biochemical changes, prior to but associated with contaminant induced toxicity, would be most useful for evaluating the sensitivity of a species.

Cytochrome P450 1A1 (CYP1A1) is an enzyme that is induced by and metabolizes planar halogenated aromatic hydrocarbons (PHAHs) such as the non-*ortho* and mono-*ortho* PCBs and polycyclic aromatic hydrocarbons (PAHs). The CYP1A1

induction pathway has been well documented in mammals (Whitlock 1999) and at least a partial sequence of the CYP1A1gene is known for multiple marine mammal species (Teramitsu *et al.* 2000). Either immunoblot or catalytic function, usually with the ethoxyresorufin-O-deethylase (EROD) activity assay, are most often used to measure CYP1A1 induction. CYP1A1 induction by PHAHs has been correlated to higher order effects such as thymic atrophy and weight loss in rats (Safe 1986). Measurements of CYP1A1 induction and correlations of CYP1A1 and contaminants in multiple species should allow us to estimate the relative sensitivity of cetacean species to PHAH toxicity.

The range of the Atlantic white-sided dolphins (*Lagenorhynchus acutus*) includes the northeast coast of the United States and Canada. This region is densely populated and has high shipping traffic. These coastal animals feed at a high trophic level and previous studies have shown that white-sided dolphins from this region have total PCB concentrations of 13 $\mu g\ g^{-1}$ wet weight in blubber (Weisbrod *et al.* 2001), similar to that seen in white-sided dolphins from the United Kingdom (Law *et al.* 2001). This concentration of PCBs was similar to that seen in harbour porpoise (Westgate *et al.* 1997) but higher than that seen in pilot whale (Weisbrod *et al.* 2001) from the same region. Based on the similarity between PCB concentrations in white-sided dolphin and concentrations reported to alter immune function in seals (Ross *et al.* 1995; de Swart *et al.* 1996), it has been suggested that the potential for toxic effects exists in this species (Weisbrod *et al.* 2001).

The white-sided dolphin is a species that commonly strands on Cape Cod, MA. Two large mass stranding events in 1998 ($\approx$ 100 white-sided and common dolphins) and 1999 ($\approx$ 60 white-sided dolphins), as well as a smaller event in 1997, allowed for the rare comprehensive collection of internal organs from multiple males and females from the same species. Here we report the expression of CYP1A1 in various internal organs and the relationship of this expression to the levels of contaminants in blubber.

Materials and Methods

Samples

A total of 12 dolphins, 5 males and 7 females, were sampled for this study from white-sided dolphins during mass strandings on Cape Cod during 1997, 1998 and 1999 (Table 1). Morphometric information, including total length and girth, gender, and reproductive status were recorded for each animal. Body length was used as a proxy for sexual maturity, with females < 206 cm, and males > 212 cm considered sexually mature (Sergeant *et al.* 1980; Palka *et al.* 1997). Multiple teeth, usually 2 teeth from the middle of each of the upper and lower jaw, were collected for age determination. Adrenal, brain, heart, intestine, kidney, liver, lung, lymph node, mammary, muscle, ovary, spleen, stomach, testis, thymus and uterus were sampled whenever possible. One portion of each organ was flash frozen in liquid nitrogen during field necropsies. Approximately 300 g of blubber was taken just anterior and slightly ventral to the dorsal fin. The blubber samples extended the entire depth of the dermis, from the epidermis to the underlying muscle layer. Blubber samples were taken and archived at the National Institute for Standards and Technology (NIST, Charleston, SC) for contaminant analyses according to established NIST protocols (Becker *et al.* 1999). Blubber samples were placed on ice and stored -20°C until shipped to NIST, where they were transferred to -150 °C liquid nitrogen (LN_2) vapor freezers. All necropsies took place within an estimated 24 hours after death. Some of these animals were euthanized.

Age Determination

Ages were determined for 5 dolphins by measurement of dentine growth layers (Scheffer & Myrick, 1980). Teeth were fixed in 10 % neutral buffered formalin and processed as described elsewhere (Tuerk 2002). Thin sections (25 μm) were made using a BFS series freezing stage (Physitemp, Clifton, NJ) connected to a SM2000R microtome (Leica, Nussloch, Germany). The tooth was sectioned on the horizontal plane until sections passed through the center of the pulp cavity. Sections stained with hematoxylin and dentine growth layers were counted at least three times each by two readers and an average age estimate was calculated. Layers of dentine stain either darkly, or do not accept stain, thus creating a pattern of alternating light and dark bands. One light and one

dark layer typically represent 1 year of growth (Scheffer and Myrick 1980). Studies using *T. truncatus* of known age have shown that this pattern is consistent and reliable proxy for determining age (Scheffer *et al.* 1980; Hohn *et al.* 1989).

Preparation of Microsomes

Approximately 1 gram of tissue was homogenized with a Teflon-glass homogenizer (10 passes) in 6 ml cold homogenization buffer (50 mM Tris, 0.15 M KCl, pH 7.4). Homogenates were centrifuged at 750×g for 10 min and then at 12 000×g for 10 min. The supernatant was removed and centrifuged for 70 min at 100 000×g. The resulting pellet was resuspended in 1 ml TEDG buffer (50 mM Tris-HCL, 1 mM EDTA, 1mM dithiothreitol, 20% (v/v) glycerol, pH 7.4) and frozen in liquid nitrogen. Microsomal protein concentrations were determined with the bicinchoninic acid assay (Smith *et al.* 1985) adapted for a 96-well plate reader, using bovine serum albumin (Sigma, St. Louis MO) as the standard.

Cytochrome P450 and b5

The content of liver microsomal cytochrome P450 was determined from dithionite difference spectra of CO-treated samples (Stegeman *et al.* 1979), assuming extinction coefficients of 91 mM^{-1} cm^{-1} for P450 and 111 mM^{-1} cm^{-1} for P420 (Omura and Sato 1964). Cytochrome b5 was measured using NADH difference spectra, assuming an extinction coefficient of 185 mM^{-1} cm^{-1}, as described previously (Stegeman *et al.* 1979).

Enzyme Assays and Immunoblots

NADPH cytochrome c (P450) reductase activity was measured by monitoring absorbance at 550 nm, as previously described (Stegeman *et al.* 1979). Ethoxyresorufin O-deethylase (EROD) activity was quantified using a spectroflurometric kinetic assay (Kennedy *et al.* 1993). Immunoblot (western blot) analyses were performed as previously described (Kloepper-Sams *et al.* 1987; Stegeman *et al.* 1991) with an enhanced chemiluminescent detection system (Matthews *et al.* 1985). The primary and secondary antibodies were the monoclonal antibody 1-12-3 (3 μg ml^{-1} in 5% nonfat dry milk, 0.02 M Tris, 0.5 M NaCl, pH 7.5) and a goat anti-mouse horseradish peroxidase (BioRad, Richmond CA, 1 in 1000 dilution in 5% nonfat dry milk, 0.02 M Tris, 0.5 M

NaCl, pH 7.5), respectively. CYP1A1 content was quantified by comparison of the intensities of individual bands with the linear response range of liver microsomes from β-napthoflavone injected scup (*Stenotomus chrysops*), with known CYP1A1 content. Immunoblot (slot blot) analyses were perfomed with a Milliblot-S (Millipore, Bedford MA) apparatus with ≤ 7 μg microsomal protein applied directly to a 0.2 μm nitocellulose sheet (Protran BA 83, Scleicher and Schuell, Keene NH). Wells were washed with phosphate buffered saline and then processed as described for western blotting. CYP1A1 content was estimated densitometrically from developed X-ray audiograms of blots. Because cross-reactivity of the primary antibody for CYP1A proteins in different species may vary, CYP1A1 concentrations deduced from the immunoblots represent relative rather than absolute concentrations for dolphins.

Blubber Contaminants

Blubber samples were cryohomogenized according to established procedures (Zeisler *et al.* 1983; Becker *et al.* 1999). Sample extraction has been described in detail elsewhere (Kucklick *et al.* 2002). Briefly, samples were mixed with $NaSO_4$ and added to a pressurized fluid extraction cell (PFE) along with an internal standard solution containing 4,4'-DDT-d_8, 4,4'-DDE-d_8, 4,4'-DDD-d_8, Endosulfan I-d_4, PCB 103 and PCB 198, and were extracted with CH_2Cl_2 using PFE.

The sample extracts were reduced to between 0.5 mL and 1 mL by evaporation in a stream of purified N_2 using a Turbovap II (Zymark, Hopkinton, MA), and cleaned-up using size-exclusion chromatography using a 600 mm x 25 mm (10 □m particle size with 100 Å diameter pores) PLGel column (Polymer Labs) with CH_2Cl_2 as the mobile phase. The extract was then fractionated using a semi-preparative aminopropylsilane column (μBondapak NH_2, Waters) into relatively lower and higher polarity fractions (F1 and F2, respectively)

PCB and chlorinated pesticides (excluding toxaphene) were determined using a Agilent 6890/5973 (Palo Alto, CA) gas chromatography – Mass spectrometry (GC-MS) operating in the electron impact mode. Samples were injected on column (2 μL) onto a 60 m DB-5ms (J&W Scientific, Folsom, CA) capillary column (0.25 mm i.d. x 0.25 μm

film thickness). Helium was used as the carrier gas at a constant flow rate of 30 cm s^{-1}. The F1 and F2 from the HPLC column were injected separately using individual selected ion monitoring (SIM) programs targeting only the analytes in each fraction. For F1, the initial column temperature was 60°C; the temperature was then ramped to 150°C at 25°C min^{-1}, then to 200°C at 0.75°C min^{-1}. The final ramp brought the temperature to 300°C at 2°C min with a 10 min hold. For F2, the initial column temperature was 60°C; the temperature was increased to 170°C at 25°C min^{-1}, then to 200°C at 1°C min^{-1}, then to 240°C at 2°C min^{-1}. The final ramp brought the temperature to 300°C at 10°C min^{-1}, with a 10 min hold.

Toxaphene congeners were determined using the Agilent 6890/5973 GC-MS operating in the negative chemical ionization mode (NCI). Two calibration curves were prepared for total toxaphene using NIST SRM 3067 (Technical Toxaphene in methanol), as well as four specific toxaphene congeners (2-endo, 3-exo, 5-endo, 6-exo, 8, 8, 10, 10-octachlorobornane (TOX 26), 2-endo, 3-exo, 5-endo, 6-exo, 8, 8, 9, 10, 10-nonachlorobornane (TOX 50), and 2, 2, 5, 5, 8, 9, 9, 10, 10-nonachlorobornane (TOX 62) and toxaphene component 32 (2-endo, 3-exo, 6-exo, 8, 9, 10, 10-heptachlorobornane) (Promochem, Germany). F1 and F2 were recombined prior to toxaphene analysis since it was determined that toxaphene eluted in both fractions. Samples were injected (2 µL) onto the 60 m DB-5ms capillary column. Helium was used as the carrier gas at a constant flow rate of 30 cm s^{-1}. Methane was used as the reagent gas. The source temperature was held at 136°C. The initial column temperature was 60°C; the temperature was then ramped to 150°C at 25°C min^{-1}, then to 200°C at 0.75°C min^{-1}, then to 240°C at 2°C min^{-1}. The final ramp brought the temperature to 300°C at 10°C min^{-1} with a 10 min hold.

A calibration solution containing polybrominated diphenyl ether (PBDE) congeners BDE 47 (2, 2', 4, 4'-tetraBDE), BDE 99 (2, 2', 4, 4', 5-pentaBDE), BDE 100 (2, 2', 4, 4', 6-pentaBDE), BDE 153 (2, 2', 4, 4', 5, 5'-hexaBDE), BDE 154 (2, 2', 4, 4', 5, 6'-hexaBDE), and BDE 183 (2, 2', 3, 4, 4', 5', 6-heptaBDE), was used to determine PBDE concentrations. PBDE congeners were determined using an Agilent 5890/5972 GC-MS operating in the electron impact mode. Samples were injected (2 µL, splitless

injection) onto a 30 meter DB-5ms capillary column (0.25 mm i.d. x 0.25 μm film thickness). The injector temperature was 260°C/min. Helium was used as the carrier gas at a constant flow rate of 30 cm s^{-1}. The initial column temperature was 80°C with a two-min hold. The temperature was then ramped to 200°C at 30°C min^{-1}, then to 300°C at 5°C min^{-1} with a 10 minute hold. The ion source temperature was 250°C.

The amount of analyte present was determined using the slope and intercept of calibration curves established for each suite of contaminants. Analyte concentrations were determined using the following internal standards: PCB 103, PCB 198, or 4,4'DDD-d_8 for all PCBs and chlorinated pesticides concentrations and PCB 204, PCB 198, or ^{13}C - BDE 99 for PBDEs. Detection limits for all PCBs and most pesticides were 2 ng g^{-1} wet mass. Detection limits were100 ng for total toxaphene, 30 ng for individual toxaphene congeners, and 2 ng for PBDE congeners.

Chlorinated pesticides measured included hexachlorobenzene (HCB); α-, β-, and γ -hexaclorocyclohexane (HCH); heptachlor and heptachlor epoxide; trans- and cis-chlordane; trans- and cis- nonachlor; oxychlordane; endosulfan I; dieldrin; 2,4′-, and 4,4′-DDD; 2,4′-, and 4,4′-DDE; 2,4′-, and 4,4′-DDT; mirex; total toxaphene and toxaphene congeners 26, 32, 50, and 62; and PBDE congeners 47, 99, 100, 153 and 154. Total PCB concentration (ΣPCB) was calculated as the sum of individual peaks for all 56 *ortho*-substituted PCBs analyzed (IUPAC# 18, 28, 29, 31, 44, 45, 49, 52, 56, 60, 63, 66, 70, 74, 76, 81, 82, 84, 87, 89, 90, 92, 95, 99, 101, 105, 107, 110, 118, 123, 128, 132, 138, 146, 149, 151, 153, 154, 156, 157, 158, 163, 170, 171, 174, 180, 182, 183, 187, 193, 194, 195, 201, 202, 206, 209). Total DDT concentration (ΣDDT) was calculated as the sum of the DDT compounds (2,4′-, and 4,4′- DDD, DDE, and DDT) and total PDBEs (ΣPBDE) was calculated as the sum of the PBDE congeners (26, 32, 50, 62). The Σmono-*ortho* PCB concentration was calculated as the sum of individual peaks of 17 mono-*ortho* substituted PCB congeners (IUPAC# 29, 28, 31, 56, 60, 63, 66, 70, 74, 76, 81, 105, 107, 118, 123, 156, 157) and four *ortho* substituted PCBs (IUPAC # 87, 149, 171, 202) that co-eluted with a mono-*ortho* substituted PCB.

Results

A total of 12 white-sided dolphin, 7 females and 5 male, were collected during field necropsies of mass strandings on Cape Cod, MA in 1997, 1998 and 1999 (Summary information in Table 1). At least half of these animals were euthanized, which gave a precise time of death. The dolphins were necropsied within an estimated twenty-four hours post-mortem, although most were necropsied within twelve hours and two within three hours. Five animals were considered sexually mature, based on their lengths. Both females that were considered sexually mature were lactating. Ages were determined for only 5 animals, based on the availability of teeth, but known ages agreed with assignment of sexual maturity by length and rough estimates on age determined by published length-age curves (Sergeant *et al.* 1980). Rough estimates of age suggest that females and males probably ranged from 2 to 24 years and 2 to 10 years, respectively (data not shown). However, except for two females, all other dolphins were less than 10 years of age.

Concentrations of contaminants in blubber were determined for nine animals included in this study. Blubber concentrations were determined for 56 PCB congeners; HCB; α-, β-, and γ -HCH; heptachlor and heptachlor epoxide; trans- and cis- chlordane; trans- and cis- nonachlor; oxychlordane; endosulfan I; dieldrin; 6 DDT congeners; mirex; total toxaphene and 4 toxaphene congeners and 5 PBDE congeners. A detailed analysis of contaminant concentrations in white-sided dolphins from this region, including these samples, has been given elsewhere (Tuerk 2002). PCB concentrations (total and Σmono-*ortho* congeners) do not seem to have a strong relationship with length in either males or females (Figure 1 and 2), and transforming the data using the natural logarithm did not improve the relationship. This is probably reflective of the small sample size for each sex. Length – PCB relationships had a negative slope using a larger data set than in this study (Tuerk 2002). Total PCB concentrations averaged 25 $\mu g\ g^{-1}$ (±82.6 SD) and 18.5 $\mu g\ g^{-1}$ (±3.6 SD) for males and females, respectively.

Total P450, P420 and cytochrome b5 contents in liver samples are shown in Table 2. Total P450 ranged from 0.05 to 0.191 nmol mg^{-1} but most samples had 0.01 to 0.02 nmol mg^{-1} (Table 2). A significant P420 peak was present in all of the liver microsome

samples (Table 2), accounting for greater than 50% of the total P450 and P420 content. The relative P420 content was not related to the time between death and necropsy (Figure 3). NADPH cytochrome c (P450) reductase was measured in kidney, liver and lung (Table 3) and was always higher in liver than the other two organs. The mean reductase activity was 120.3 (±15.5 SD), 237.6 (±75.1 SD), and 122.3 (±13.6 SD) nmol mg^{-1} min^{-1} for kidney, liver, and lung, respectively. There was no statistical difference between reductase activities in the kidney and lung, but reductase activity was significantly greater in the liver than the other two organs.

There was no correlation between the EROD activity in liver and the relative P420 content in the liver (Figure 4) or the time between necropsy and death (Figure 5). EROD activity was highest in liver, ranging from 9.2 to 376 pmoles mg^{-1} min^{-1}, while all other internal organs had EROD activities less than 13 pmoles mg^{-1} min^{-1} (Table 4). Similar to EROD activities, CYP1A1 contents, as measured by immunoblot, were low. Microsome samples usually had CYP1A1 levels less than 3 pmol mg^{-1} in all internal organs. Contrary to EROD activities, where the highest activities were all in liver, CYP1A1 content was not higher in liver than other organs and the highest concentrations (21.8, 8.17, and 5.67 pmoles mg^{-1}) were recorded in two lung and one spleen sample, all from different animals. CYP1A1 and EROD activity were not well correlated, no matter whether all organs ($r=0.02$, $p>0.05$, Figure 6) or just liver samples ($r=0.38$, $p>0.05$, Figure 7) were included in the analysis.

Liver CYP1A1 content was not well correlated with either total or Σmono-*ortho* PCBs (Figure 8) but was significantly correlated with PCB70, PCB156 and PCB157, as well as HCB (Table 5). Liver EROD activity was not well correlated with total or Σmono-*ortho* PCBs (Figure 9), any individual mono-*ortho* PCB congener, or any of the chlorinated pesticides when all liver samples were included in the analysis. The sample (99-018) with the highest EROD activity was over 3 times higher than the next highest liver EROD and three standard deviations above the mean liver EROD activity. This sample also had higher total P450 and P420 content and reductase activity, compared to other samples. It was clearly an outlier in this data set. When this sample was dropped

from the analysis, EROD activity was significantly correlated with both total and Σmono-*ortho* PCBs, as well as several individual mono-*ortho* PCB congeners (CB66, CB107, and CB149) and heptachlor (Table 5). The removal of the outlier did not improve the relationship between CYP1A1 content and any contaminant concentration, but the correlation with HCB was no longer significant.

Discussion

Condition and Life-History of Animals

The reasons for mass strandings of dolphins and whales are unknown, although in recent years there have been several cases of large mortality events related to epizootic outbreaks. Even so, the reasons for most mass stranding events, when many individual cetaceans strand at the same time and geographic location, are unclear and the animals often appear healthy and robust. Mass strandings of cetaceans are not unusual on Cape Cod, MA and have included pilot whale, common dolphin and Atlantic white-sided dolphin (Sergeant *et al.* 1980; Wiley *et al.* 2001). The animals involved in the 1997, 1998 and 1999 mass strandings, when sampling for this study took place, were apparently healthy, robust animals and there is currently no known cause of these strandings. These samples should be representative of the normal, healthy free-ranging population of Atlantic white-sided dolphin in the northwest Atlantic Ocean.

To obtain tissues that were not degraded, animals selected for sampling were those thought to have most recently died. Time between death and necropsy varied between samples because the exact time of death was not available prior to necropsy, due to logistical issues. In all years, the strandings occurred during winter when cooler air temperatures would have helped to minimize degradation during the collection of samples. Necropsies were performed in the field under difficult circumstances, so that sampling was not identical for each animal. In spite of this, a large group of internal organs, including adrenal, heart, kidney, liver, lung, lymph node, spleen, and gonads, were collected from most or all animals.

The unavailability of teeth limited our ability to determine ages from dentine growth layers. Only 5 animals could be aged in this manner. Although morphometric

measurements were taken, published age – length relationships in this species did not include a mathematical model (Sergeant *et al.* 1980) which we could apply to our length data. Rough estimates of age, based on the published data and which agree with the few animals we could age by examining teeth, suggest that intermediate age dolphin were not well represented in our samples. Mass strandings of white-sided dolphins involve herds whose composition is biased towards young and old animals (Sergeant *et al.* 1980). Based on the age at separation from the breeding herd (Sergeant *et al.* 1980), the youngest animals (age two years) may have been offspring of females in the herd from the prior breeding season (i.e. not young of the year). These animals were likely consuming the same food resources as the other stranded animals and not consuming milk (Sergeant *et al.* 1980).

Based on the length at maturity, two females and three males were sexually mature. In agreement with this, both females that were considered sexually mature, but no other females were lactating. Since females give birth from May to August, the sexually mature, lactating animals had most likely been lactating for approximately five to seven months, or approximately one-third to one-half of the lactation period (Sergeant *et al.* 1980). The lactating females did not appear to be different in any measurement from the other animals in this study, except in having lower PCB concentrations than other females, and were therefore included in all analyses.

Characterization of Microsomal Systems

For appropriate catalytic function, cytochrome P450 enzymes receive electrons through NADPH-cytochrome c (P450) reductase or cytochrome b5. Reductase activities in liver microsomes were greater that those seen in liver microsomes from beluga whale (White *et al.* 1994), killer whale, short finned pilot whale, striped dolphin (Watanabe *et al.* 1989), and minke whale (Goksoeyr *et al.* 1986; Goksoeyr *et al.* 1988). Reductase activities in kidney and lung were higher than those reported for liver in all other cetaceans except striped dolphin (Watanabe *et al.* 1989) and 10 times higher than those reported for minke kidney microsomes (Goksoeyr *et al.* 1988). Cytochrome b5 content was similar to or less than that seen in liver microsomes from beluga whale (White *et al.* 1994), pilot whale (White *et al.* 2000), and minke whale (Goksoeyr *et al.* 1986; Goksoeyr

et al. 1988) although it did vary 5 fold between samples. The reductase activities and cytochrome b5 contents suggest a similar or greater capacity for electron transfer to P450 enzymes in white-sided dolphin, than other cetacean species.

Although there were significant amounts of P420 present in all of the liver microsome samples, the relative P420 content did not relate to either EROD activity nor time from death to necropsy (Figures 3 and 4). This suggests that the P420 peak may have been caused by interfering compounds such as oxyhemoglobin and was not degraded P450. Significant P420 peaks were present in beluga whale liver microsomes even though the assays were performed in the presence of ascorbic acid and phenazine metosulfate (White *et al.* 1994), which is meant to remove oxyhemoglobin (Johannesen and DePierre 1978). Total P450 content for most dolphins was 0.01 to 0.02 nmol mg^{-1}, amounts which are ~ 10 - 20 fold less than those in liver of beluga (White *et al.* 1994), pilot whale (both long and short-finned species, Watanabe *et al.* 1989; White *et al.* 2000), killer whale, and striped dolphin (Watanabe *et al.* 1989), and up to 35 fold times less than those in minke whale liver (Goksoeyr *et al.* 1986; Goksoeyr *et al.* 1988). Even if all of the P420 was degraded P450, total P420 and P450 content is still less than that seen in beluga and minke whale, except for dolphin 99-018, which had much higher P450 content, P420 content, reductase activity, and EROD activity in liver microsomes than all the other animals. Any reductions in a specific P450 activity, compared to other cetacean species, are most likely directly related to the difference in the content of the enzyme, and not insufficient electron transfer.

CYP1A1 Expression in White-sided Dolphin

EROD activity was significantly greater in the liver than in any other organ as expected. Mean liver EROD activity was 90.3 pmol mg^{-1} min^{-1}, which was similar to that reported in pilot whales (both long and short-finned species, Watanabe *et al.* 1989; White *et al.* 2000) and female beluga whale (White *et al.* 1994). Liver EROD activities were much less than those reported for male beluga whale (White *et al.* 1994), minke whale (Goksoeyr *et al.* 1986; Goksoeyr *et al.* 1988), striped dolphin, killer whale (Watanabe *et al.* 1989), and sperm whale (Boon *et al.* 2001). Liver EROD activities in white-sided

dolphin are amongst the lowest reported for cetacean species. Only sperm whale and white-beaked dolphin have lower EROD activities (Boon *et al.* 2001).

Interpretation of EROD activities in extrahepatic organs is difficult since only one study has included any extrahepatic organs. Mean EROD activity in minke whale kidney was 20 pmoles mg^{-1} min^{-1} (Goksoeyr *et al.* 1988), a value similar to the highest EROD activity found in extrahepatic organs from white-sided dolphin. Although the highest EROD activity in white-sided dolphin was similar to that reported in minke kidney, most organs had lower EROD activity.

Initial immunoblots (western blots) indicated that CYP1A1 content was low in these samples and could not be detected in most organs with sample loading of ≤ 200 µg per lane. A single band, of similar molecular weight as the scup liver standard, was visible for samples with EROD activities higher than 50 pmoles mg^{-1} min^{-1}, when protein loading was very high (60 – 200 µg per lane). Similar to these white-sided dolphin samples, CYP1A1 bands have been undetectable in western blots with large sample loading, from 100 to 200 µg protein per lane, for other white-sided dolphin and pilot whale samples (B. Woodin, pers. com.).

The determination of CYP1A1 content was performed using slot blots, where microsomal protein was directly bound to membranes without initial electrophoresis as is performed for western blotting. Although this procedure allowed for a much lower limit of detection than western blots, many of these samples were so low that results were more variable. The large dilution needed for this assay probably contributed to the variability between blots. Although this procedure confirmed that CYP1A1 is lowly expressed in white-sided dolphins, western blots, with large sample loadings (i.e. greater than 200 µg protein), would be needed to ensure quantitative determination of CYP1A1 expression.

CYP1A1 content in all organs was very low, which, except for liver, was expected based on such low EROD activities. CYP1A1 content has been determined in beluga and pilot whale liver microsomes (White *et al.* 1994; White *et al.* 2000) using the same antibody and standards as this study. CYP1A1 content in liver of pilot whale (White *et al.* 2000) and female beluga (White *et al.* 1994) was six to seven times higher than the

mean CYP1A1 content of most internal organs in white-sided dolphin and twice that of the highest CYP1A1 levels recorded in this study. Male beluga whales had hepatic CYP1A1 levels that were nearly 70 times higher than that reported here for white-sided dolphin (White *et al.* 1994). There will be some inherent differences in the cross-reactivity of this antibody for CYP1A1 from different species. Yet, differences in cross-reactivity could not account for such large differences in CYP1A1 content between these species, since the epitope for this antibody is well conserved and the antibody recognizes CYP1A enzymes in diverse vertebrate species (Stegeman and Hahn 1994). CYP1A1 content in white-sided dolphin liver, like EROD activity, is significantly less than that reported for other cetacean species.

Surprisingly, CYP1A1 content was not highest in the liver samples and CYP1A1 levels did not correlate well with EROD activity, even when the analysis was limited to liver. This is probably a reflection of the low levels of CYP1A1 and variability of the CYP1A1 data for the lowest samples. Certainly, a range of only 3 pmoles mg^{-1} of CYP1A1 is very small. CYP1A1 content in liver has been strongly correlated to liver EROD activity in both pilot (White *et al.* 2000) and beluga whale (White *et al.* 1994), although the range of CYP1A1 content was 10 fold larger than that seen here.

Contaminant Concentrations

Contaminant concentrations in these white-sided dolphins were similar to those previously reported for this population (Weisbrod *et al.* 2001). Mean total PCB and Σmono-*ortho* PCB concentrations were higher in this study, but more PCB congeners were measured in this study. All chlorinated pesticide concentrations (total HCH (sum of the α, β, and γ isomers), total chlordanes (sum of trans- and cis- chlordane, nonachlor, and oxychlordane), ΣDDTs, HCB, dieldrin and mirex) were very similar between the two studies (data not shown). The PCB concentrations were also similar to those reported in other species of marine mammals from the northwest Atlantic region, including pilot whale, harbour porpoise and harbour seal (Lake *et al.* 1995; Westgate *et al.* 1997; Weisbrod *et al.* 2001).

Although length was not correlated to contaminant concentrations among these 12 animals, a larger data set has shown that contaminant concentrations decrease with length in both male and female white-sided dolphin (Tuerk 2002). Since length is known to increase with age (Sergeant *et al.* 1980), contaminant concentrations are presumed to decrease with age, contrary to all other male cetaceans studied to date (Tanabe *et al.* 1987; Martineau *et al.* 1994; Westgate *et al.* 1997; Tilbury *et al.* 1999; Westgate and Tolley 1999; Ross *et al.* 2000; Weisbrod *et al.* 2000; Hoekstra *et al.* 2002). The reasons for this relationship are unknown, although it has been suggested that white-sided dolphin may selectively metabolize some PCBs and other organochlorine contaminants better than other cetacean species (Tuerk 2002).

Correlations of CYP1A1 and PCB Concentrations

PCB concentrations, including the Σmono-*ortho* PCBs were well correlated with EROD activity but not CYP1A1 content in liver of white-sided dolphin. CYP1A1 content in liver has been strongly correlated to Σmono-*ortho* PCBs in blubber of beluga whale (White *et al.* 1994). Since EROD activities and CYP1A1 content were also strongly correlated in that study (White *et al.* 1994), it is likely that EROD activity also correlated with blubber PCB concentrations in the beluga whale. The weights of the beluga in that study were much less than expected based on their length (White *et al.* 1994), which suggested that those animals had mobilized fat reserves. The white-sided dolphins in this study were robust and apparently healthy animals and did not show signs of significant mobilization of fat. The strong relationship between liver EROD activity and Σmono-*ortho* PCBs in blubber in a different cetacean species, with a different contaminant exposure, confirms that PCBs are well correlated with measures of liver CYP1A1 in cetaceans.

Inferring the Sensitivity of Cetaceans to PCBs

Measurements of the relationship between CYP1A1 and contaminants in multiple species of cetaceans could indicate relative sensitivities of the species in question. Since we know that measures of hepatic CYP1A1, either content or EROD activity, are related to Σmono-*ortho* PCBs in both beluga whale and white-sided dolphin, we can compare

measures of CYP1A1 and contaminant exposure in the two species. While CYP1A1 content and EROD activities in liver were much higher in beluga whale than in white-sided dolphin, white-sided dolphin have much higher contaminant concentrations in their blubber. Mean total PCB concentrations in the blubber of Arctic beluga are 2 to 5 $\mu g\ g^{-1}$ (Muir *et al.* 1996), while white-sided dolphins have mean concentrations ranging from 13 to 21 $\mu g\ g^{-1}$, based on published data (Weisbrod *et al.* 2001) and this study, respectively. Therefore, beluga appear to be much more sensitive than white-sided dolphin to the induction of CYP1A1 by PCBs.

For cetacean species in the northwest Atlantic, mean PCB concentrations in pilot whale (Weisbrod *et al.* 2001) are 4 to 5 times lower than those in harbour porpoise (Westgate *et al.* 1997; Westgate *et al.* 1999) and white-sided dolphin (this study and Weisbrod *et al.* 2001). These species have overlapping geographic ranges and primary prey species, although pilot whale depend more on long-finned squid, while harbour porpoise and white-sided dolphin consume primarily Atlantic herring and hake (Sergeant *et al.* 1980; Gannon *et al.* 1997a; Gannon *et al.* 1997b; Gannon *et al.* 1997c). Yet, CYP1A1 expression in pilot whale is much higher (White *et al.* 2000) than that reported in white-sided dolphin in this study. Limited immunohistochemical data in these species supports this conclusion and suggests that northwest Atlantic harbour porpoise have the lowest CYP1A1 expression of these species (C. Miller, pers. com.). The relative contribution of PAHs to CYP1A1 induction remains unaddressed because of the dearth of PAH measurements in cetaceans in general. Yet for cetaceans that live in the same region and have the same food resources, PAH concentrations may be similar. CYP1A1 expression in integument from North Atlantic right whales appears to be related to local PAH concentrations in copepods, their main prey (M. Moore, pers. comm.). Thus, small scale regional differences in PAH concentrations may be important and PAH measurements in these three species, and possibly regional prey items, are needed before we can be certain of their relative sensitivities to CYP1A1 induction.

Conclusions

Contaminant concentrations *per se* are unlikely to indicate which species will have the largest biological response. Determining CYP1A1 expression in multiple species and comparing the level of expression to known exposures will aid our understanding of the relative sensitivity of cetacean species to contaminant induced effects. The difference in CYP1A1 expression in white-sided dolphin to that of other cetacean species suggests that white-sided dolphin may be less sensitive to the toxicity of PHAH contaminants than the beluga and pilot whale and possibly more sensitive than the harbour porpoise.

Sex does not appear to be an important variable as the values for both male and female samples fall on the same EROD activity - PCB curve, as was seen in the CYP1A1 content – PCB curve with beluga (White *et al.* 1994). Furthermore, age is clearly not a variable needed for the interpretation of CYP1A1 measurements. Contrary to other species including beluga, contaminant concentrations decrease with age in male white-sided dolphin (Tuerk 2002) and yet contaminants and EROD activity are still positively correlated, as has been seen with beluga. If age were a critical factor, positive correlations would not be seen in a species with an inverse age – contaminant relationship. Therefore, while life history parameters of age and sex may be related to contaminant accumulation, the contaminant concentrations alone appear to explain the levels of CYP1A1 expression. Studies of CYP1A1 expression in other cetacean species, which include measurement of and relationships with contaminant concentrations, should allow us to determine the relative sensitivity of cetacean species to PHAH induction of CYP1A1.

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Table 1. Summary of Life History and Stranding Information for Dolphins Included in this Study.

Dolphin	NIST Identification Number	Accession Number[1]	Sex	Length (cm)[2]	Age (yr)[3]	Sexual Maturity[4,9]	Euthanized[5,9]	Time from Death to Necropsy[6]	Reproductive Status
97-228	NM7B135	MH-97-534	F	235	NA[8]	Y	Y	10	Lactating
98-001	None[7]	MH-98-414	F	227	NA[8]	Y	U	24	Lactating
98-002	NM8B232	MH-98-415	F	182	NA[8]	N	U	24	
99-014	NM10B104C	MH-99-537	F	187	2	N	N	24	
99-015	NM11B210C	MH-99-521	F	189	2	N	Y	12	
99-018	NM11B213C	MH-99-522	F	194	NA[8]	N	Y	12	
99-021	None[7]	MH-99-563	F	180	NA[8]	N	Y	12	
98-005	NM8B248	MH-98-456	M	230	7	Y	U	2	
99-013	NM10B101C	MH-99-536	M	205	2	N	N	24	
99-016	NM8B138	MH-99-520	M	243	NA[8]	Y	Y	12	
99-017	NM10B107	MH-99-519	M	225	10	Y	U	12	
99-020	None[7]	MH-99-549	M	204	NA[8]	N	Y	3	

[1] Identification number given by the New England Aquarium; [2] Total body length from snout to fluke notch,;[3] determined by counting dentine growth layers; [4] based on total length; [5] Animals that were found alive and subsequently euthanized; [6] Maximum time from death to necropsy in hours, estimated in animals that were found dead; [7] animals without blubber contaminant concentrations; [8] NA is not available; [9] N is no, Y is yes and U is unknown.

Table 2. Total P450, P420 and Cytochrome b5 Content in White-sided Dolphin Liver Microsomes.

Study ID	Cytochrome b5[1]	Total P450[1]	Total P420[1]	Relative P420 Content[2]
97-228	0.25	0.01	0.51	0.98
98-001	0.11	0.02	0.16	0.90
98-002	0.07	0.02	0.29	0.95
98-005	0.05	0.01	0.13	0.93
99-013	NM[3]	0.01	0.26	0.95
99-014	NM	0.02	0.20	0.92
99-015	NM	0.02	0.03	0.54
99-016	0.07	0.01	0.10	0.91
99-017	0.17	0.01	0.44	0.97
99-018	NM	0.08	1.61	0.96
99-020	0.11	0.00	0.28	0.98
99-021	NM	0.19	0.53	0.73

[1] nmol mg^{-1}; [2] percent of total P420 and P450; [3] NM is not measured in this sample

Table 3. NADPH Cytchrome P450 Reductase Activity in Several Organs of White-sided Dolphin. Reductase activity is reported in nmol mg protein^{-1} min^{-1}.

Sample ID	Kidney	Liver	Lung
97-228	102.7	159.4	104.3
98-001	133.7	220.6	122.8
98-002	119.3	234.5	121.8
98-005	152.1	233.1	131.3
99-013	123.0	217.6	110.3
99-014	133.9	119.1	111.9
99-015	109.9	262.9	133.2
99-016	107.7	250.1	114.5
99-017	110.7	274.6	150.9
99-018	110.1	404.3	122.1

Table 4. Ethoxyresorufin-O-deethylase Activity and CYP1A1 Content in Liver Microsomes and Σmono-*ortho* PCBs in blubber from White-sided dolphin. CYP1A1 content is measured relative to a scup standard (see material and methods for details). PCB concentrations are based on wet weight, in blubber.

Dolphin	Sex	Σmono-*ortho* PCBs (ng g^{-1})	EROD Activity (pmoles mg^{-1} min^{-1})	CYP1A1 Content (pmoles mg^{-1})
97-228	F	1557	13.2	0.397
98-001	F	NA	9.2	0.567
98-002	F	3534	50.5	0.759
99-014	F	2852	96.6	0.680
99-015	F	3069	74.5	2.879
99-018	F	3469	376.0	1.638
99-021	F	NA	85.5	0.743
98-005	M	2136	37.7	0.689
99-013	M	4320	118.0	2.152
99-016	M	2940	42.6	0.249
99-017	M	4148	113.7	0.532
99-020	M	NA	66.5	0.410

Table 5. Correlation between CYP1A1 content (pmole mg^{-1}) or EROD activity (pmole mg^{-1} min^{-1}) in liver and contaminant concentrations (ng g^{-1} wet weight basis) in blubber of white-sided dolphin. Only contaminants that correlated with either CYP1A1 content or EROD activity are shown. The only individual PCB congeners tested were the mono-*ortho* PCBs. Correlations were performed without 99-018 included in the analysis. This sample had an EROD activity that was greater than three standard deviations above the mean. Correlation coefficients are shown and significant correlations are marked with a (*).

Contaminant Concentrations	CYP1A1 Content	EROD Activity
Total PCBs	0.16	0.74 (*)
Σmono-*ortho* PCBs	0.35	0.84 (*)
Select individual PCB congeners		
tetrachlorobiphenyls –		
CB66	0.58	0.71 (*)
CB70 and CB76[1]	0.88 (*)	0.17
pentachlorobiphenyls –		
CB107	0.44	0.73(*)
CB123[2]	0.15	0.75(*)
hexachlorobiphenyls –		
CB156[3]	0.74(*)	0.59
CB157	0.93(*)	0.64
HCB	0.68	0.58
Heptachlor	0.41	0.71(*)
Total Toxaphene	0.53	0.81(*)

[1] CB70 and CB76 coeluted, [2] CB 123 coeluted with CB149, a di-*ortho* substituted PCB, [3] CB156 coeluted with CB171 and CB202, which have 3 and 4 ortho substitutions, respectively.

Figure 1. Total PCB concentrations in blubber compared to length of white-sided dolphin. PCB concentrations are the sum of 56 PCB congeners measured in this study on a wet weight basis (see materials and methods for details).

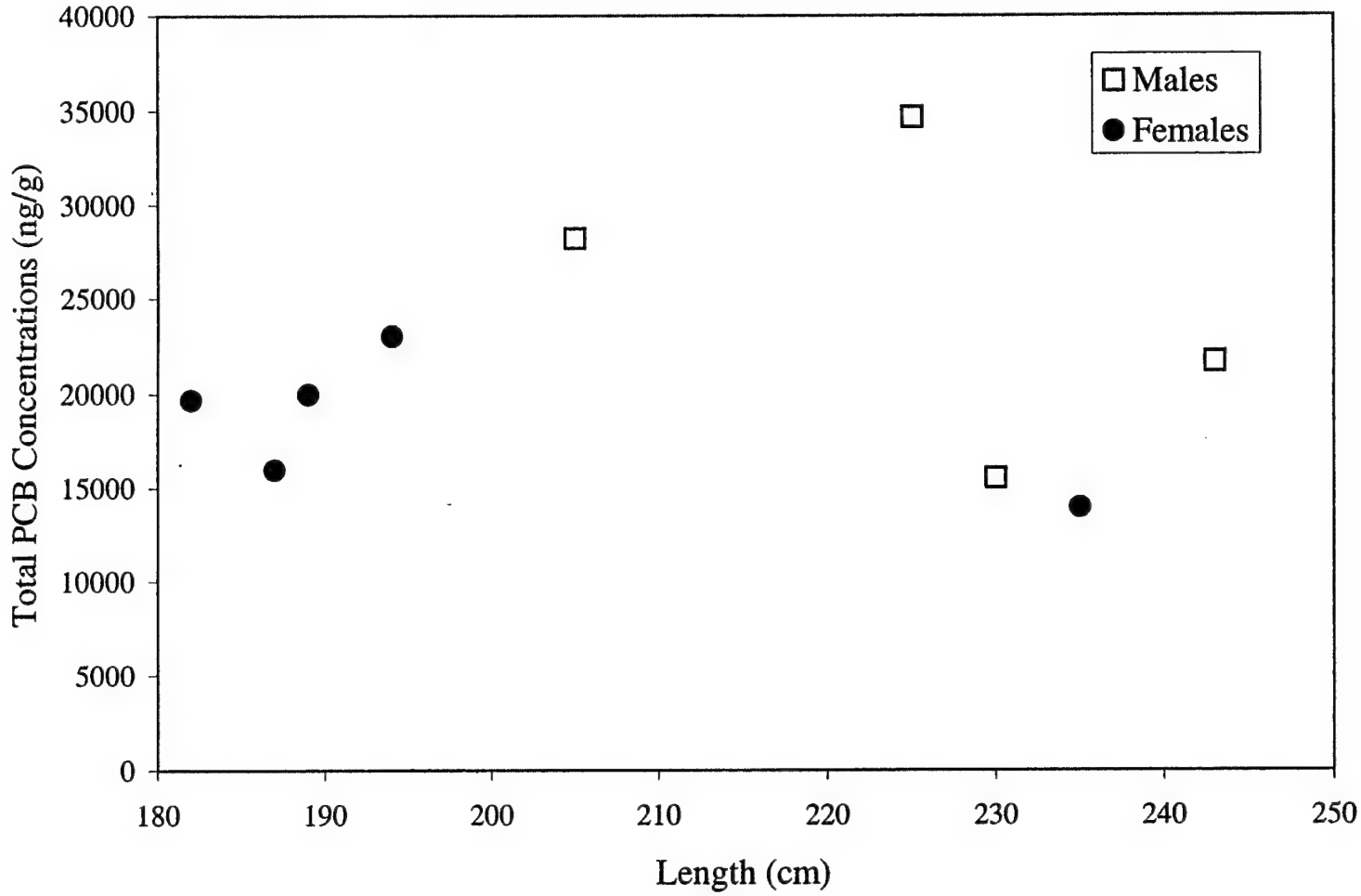

40000
35000
30000
25000
20000
15000
10000
5000
0
Total PCB Concentrations (ng/g)
180
190
200
210
220
230
240
250
Length (cm)
Males
Females

Figure 2. Σmono-*ortho* PCB concentrations in blubber compared to length of white-sided dolphin. PCB concentrations are the sum of 16 PCB congeners measured in this study on a wet weight basis (see materials and methods for details).

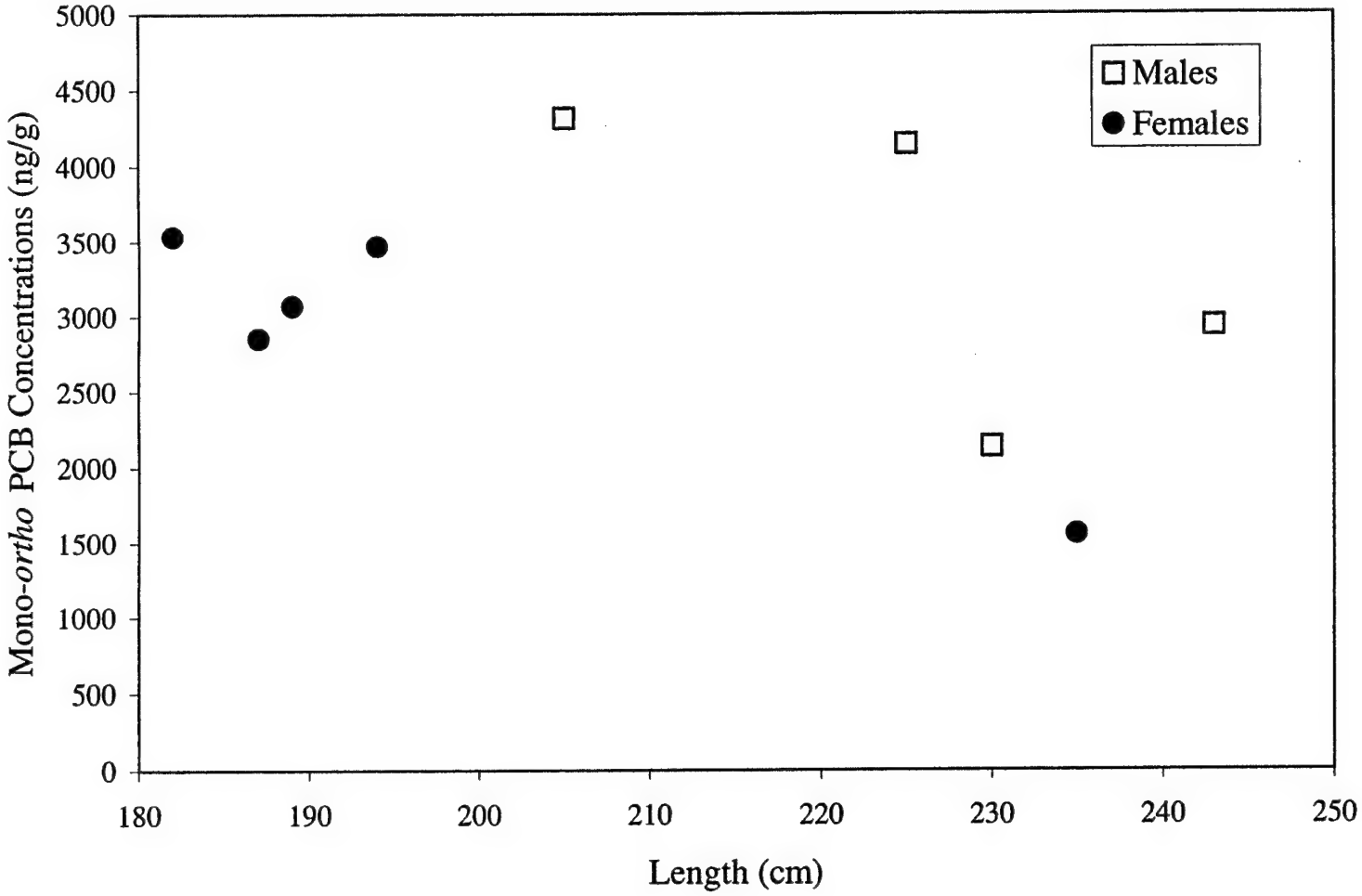

Mono-ortho PCB Concentrations (ng/g)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
180
190
200
210
220
230
240
250
Length (cm)
Males
Females

Figure 3. The relative P420 content in liver microsomes compared to the time between death and necropsy. P420 is shown as a percent of the total P450 and P420 content in each sample. Time between death and necropsy (hours) is either the known time since death or an estimate of the maximum time, for animals where the exact time of death was unknown.

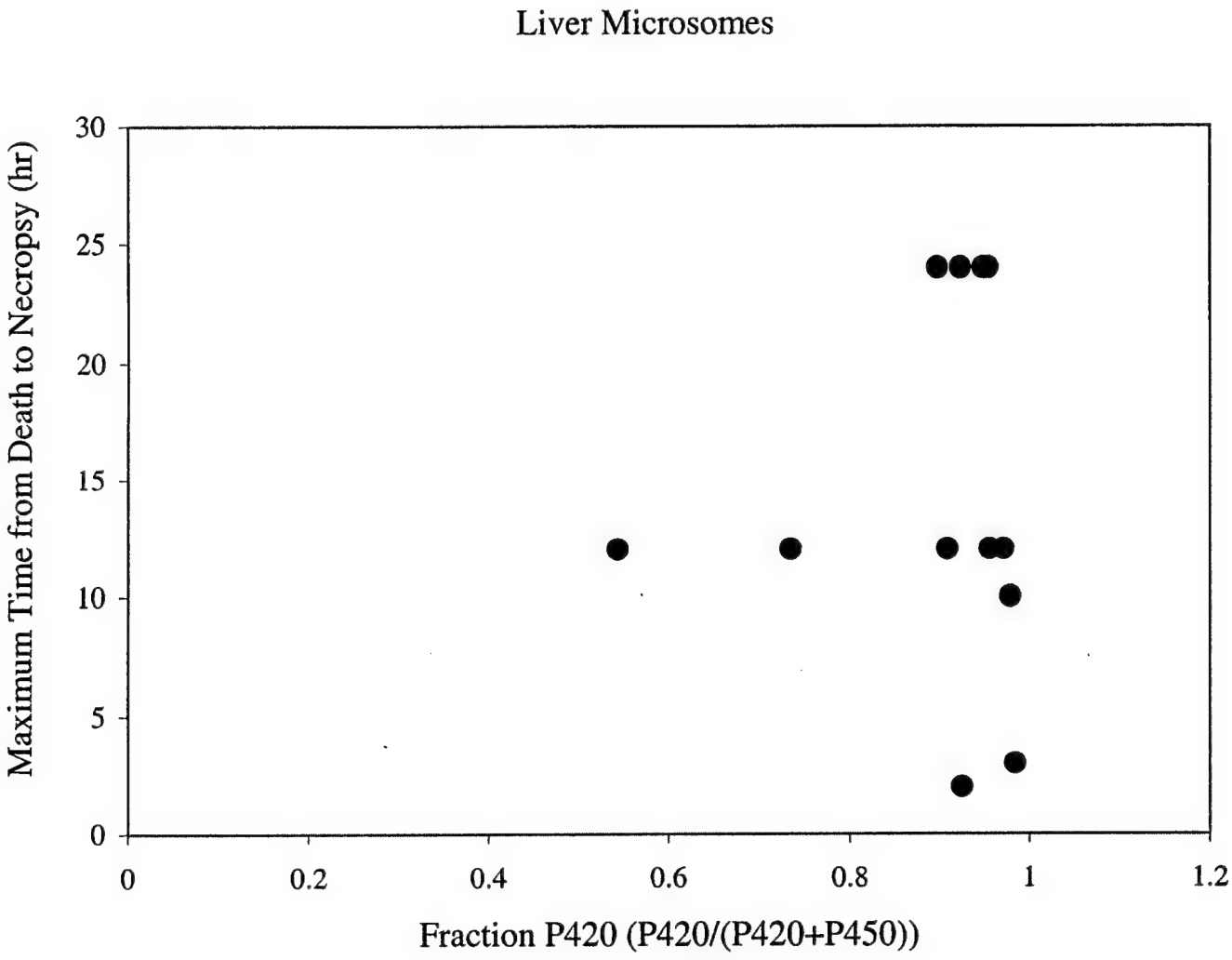

Liver Microsomes
Maximum Time from Death to Necropsy (hr)
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
Fraction P420 (P420/(P420+P450))

Figure 4. Ethoxyresorufin-O-deethylase activity versus the relative P420 in liver microsomes of white-sided dolphin. P420 is shown as a percent of both total P450 and P420 content in each sample.

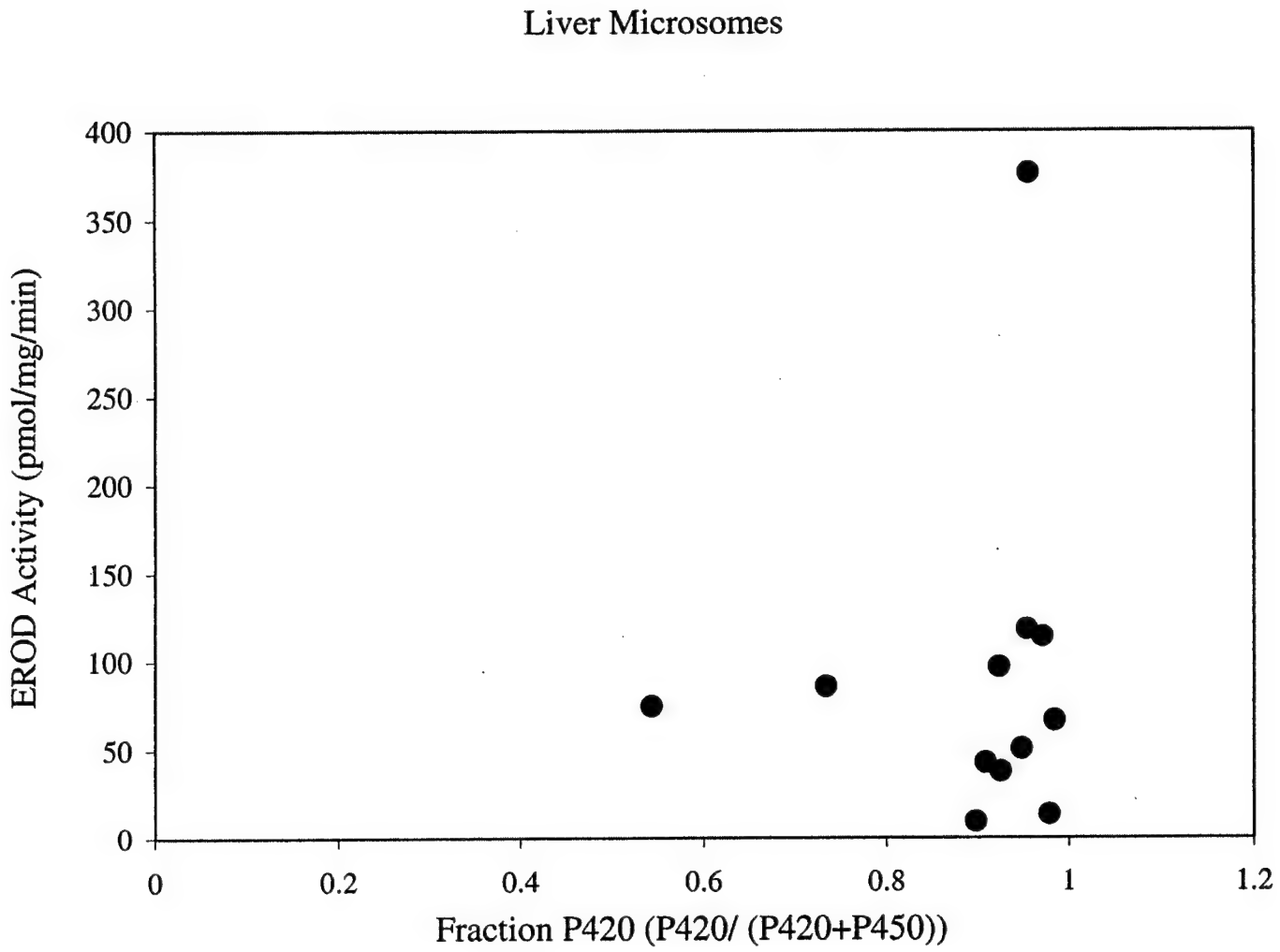

Liver Microsomes
EROD Activity (pmol/mg/min)
400
350
300
250
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1
1.2
Fraction P420 (P420/ (P420+P450))

Figure 5. Ethoxyresorufin-O-deethylase activity in liver microsomes compared to the time between death and necropsy. Time between death and necropsy (hours) is either the known time since death or an estimate of the maximum time since death, for animals where the exact time of death was unknown.

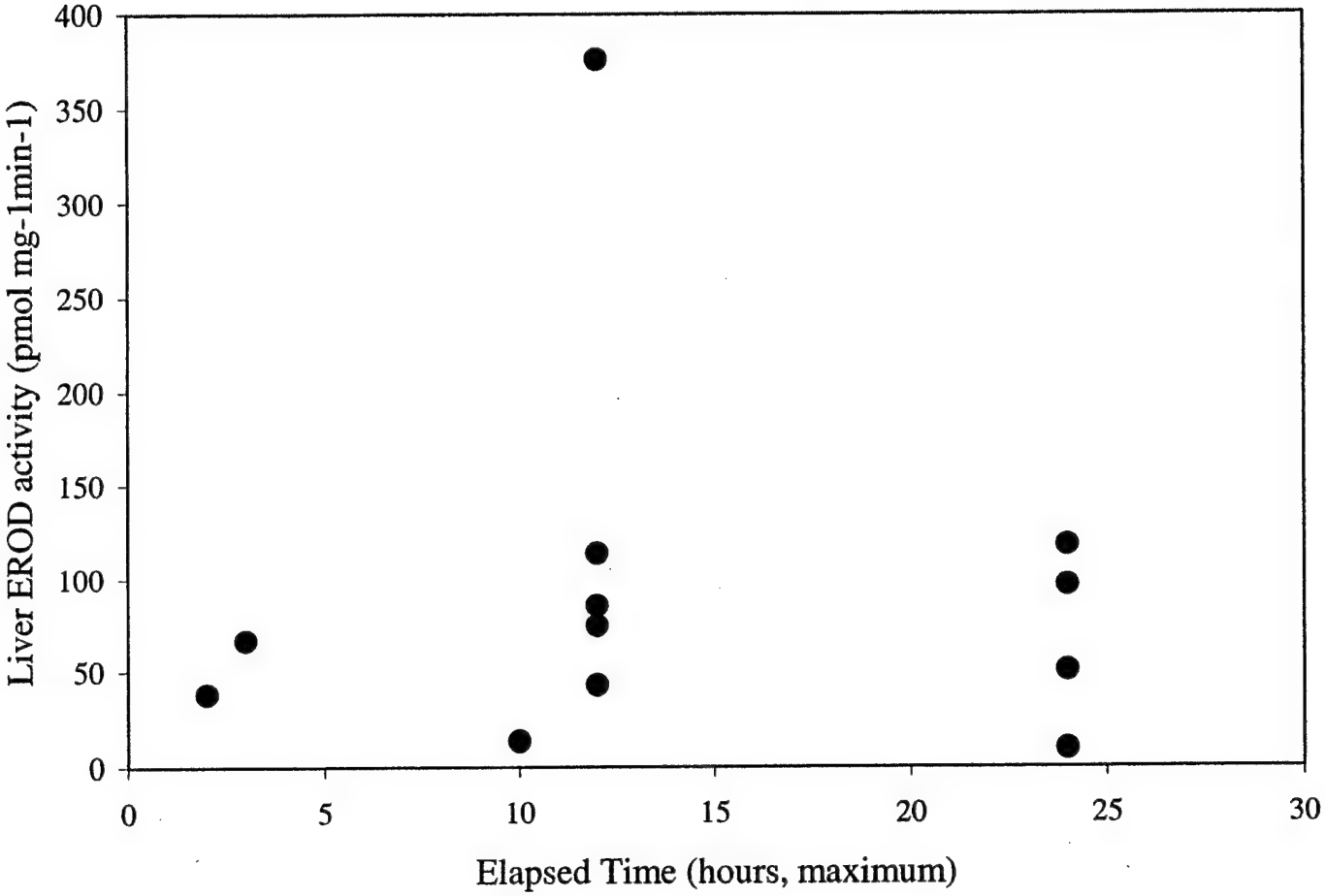

Liver EROD activity (pmol mg-1min-1)
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
Elapsed Time (hours, maximum)

Figure 6. Ethoxyresorufin-O-deethylase activity versus the CYP1A1 content in all organs of white-sided dolphin. CYP1A1 content is relative to a scup standard (see materials and methods for details).

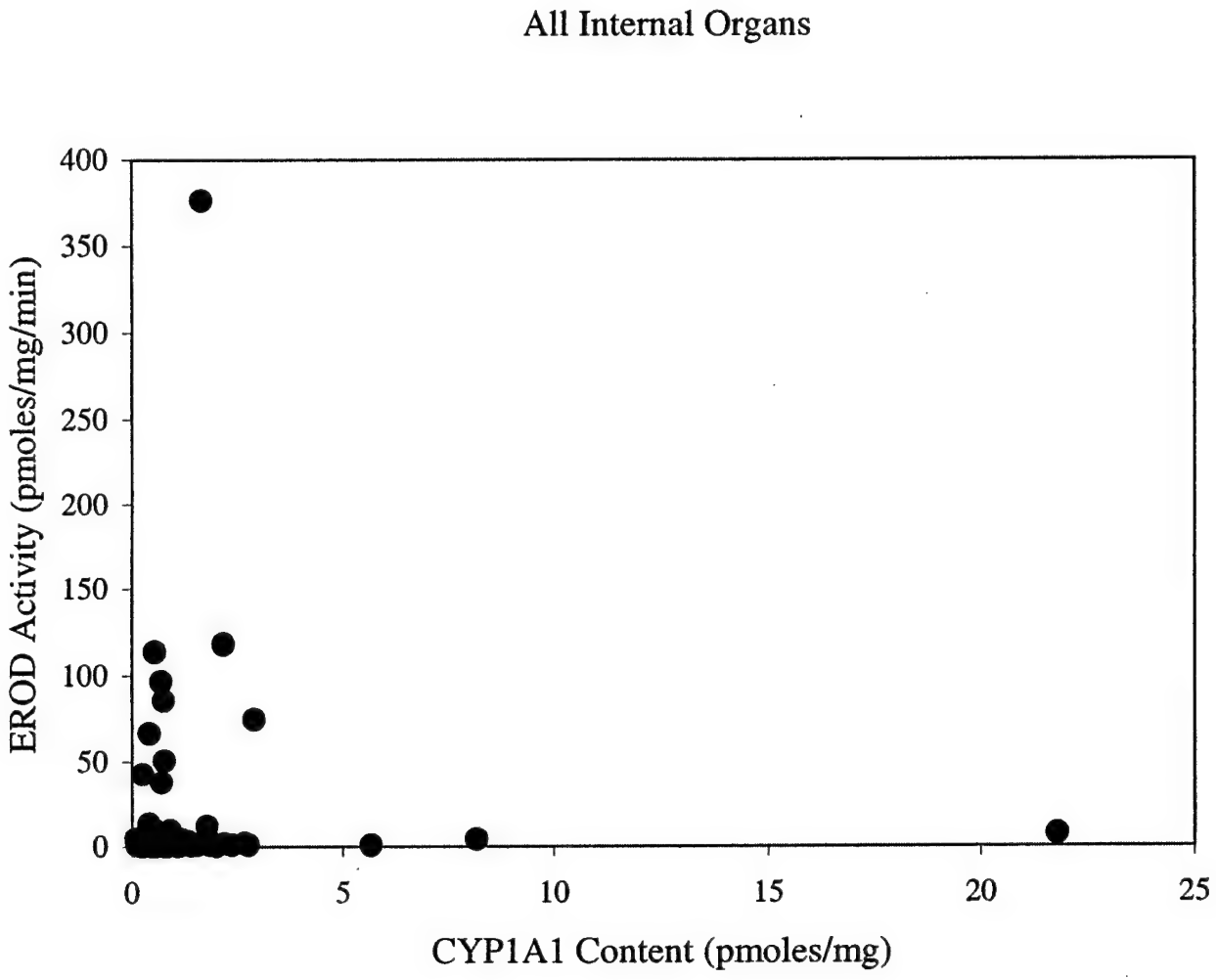

All Internal Organs
EROD Activity (pmoles/mg/min)
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
CYP1A1 Content (pmoles/mg)

Figure 7. Ethoxyresorufin-O-deethylase activity versus the CYP1A1 content in liver of white-sided dolphin. CYP1A1 content is relative to a scup standard (see materials and methods for details).

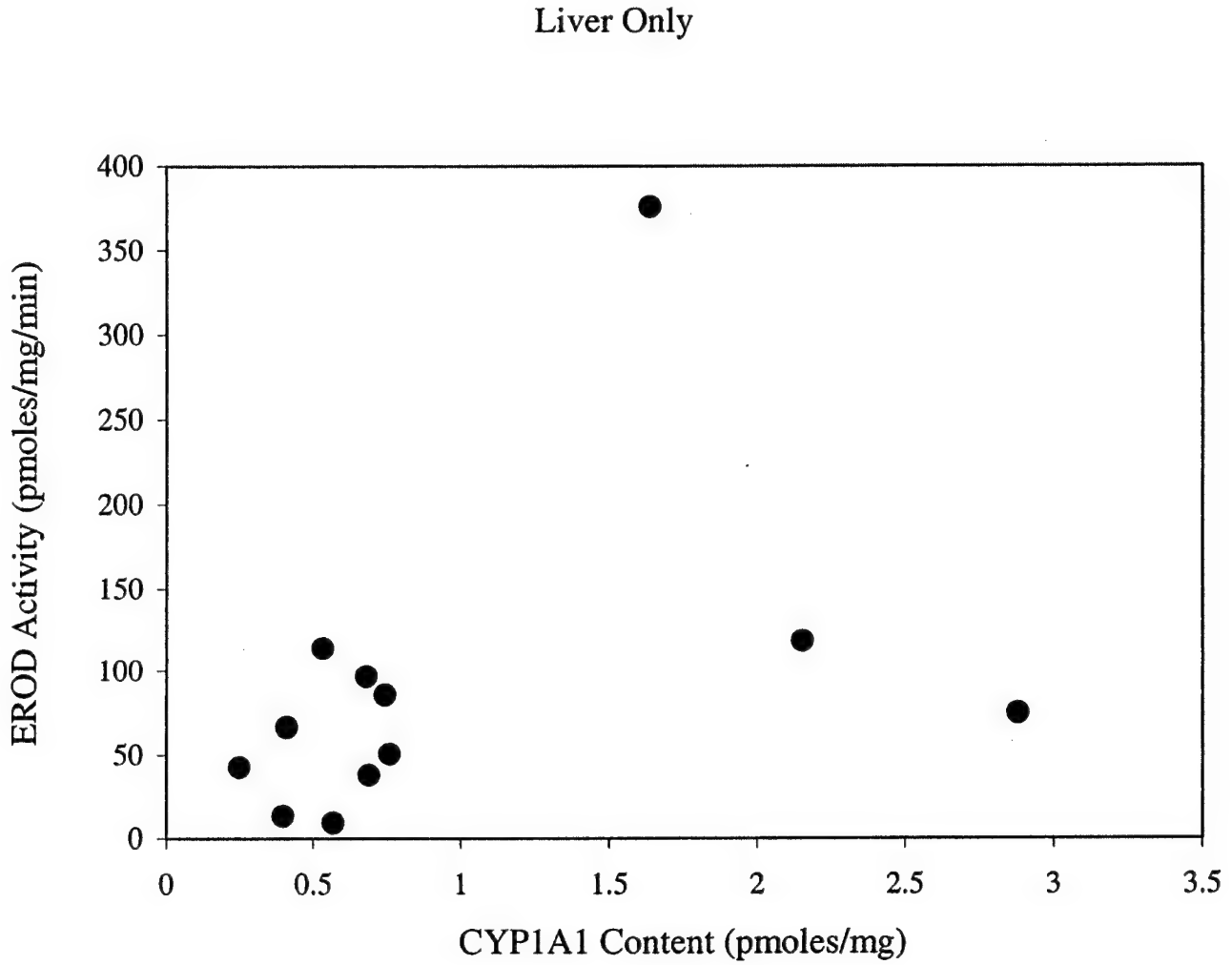

Liver Only
EROD Activity (pmoles/mg/min)
400
350
300
250
200
150
100
50
0
0
0.5
1
1.5
2
2.5
3
3.5
CYP1A1 Content (pmoles/mg)

Figure 8. Liver ethoxyresorufin-O-deethylase activity and CYP1A1 content versus the total PCB concentrations in blubber of white-sided dolphin. PCB concentrations are the sum of 56 PCB congeners measured in this study on a wet weight basis (see materials and methods for details).

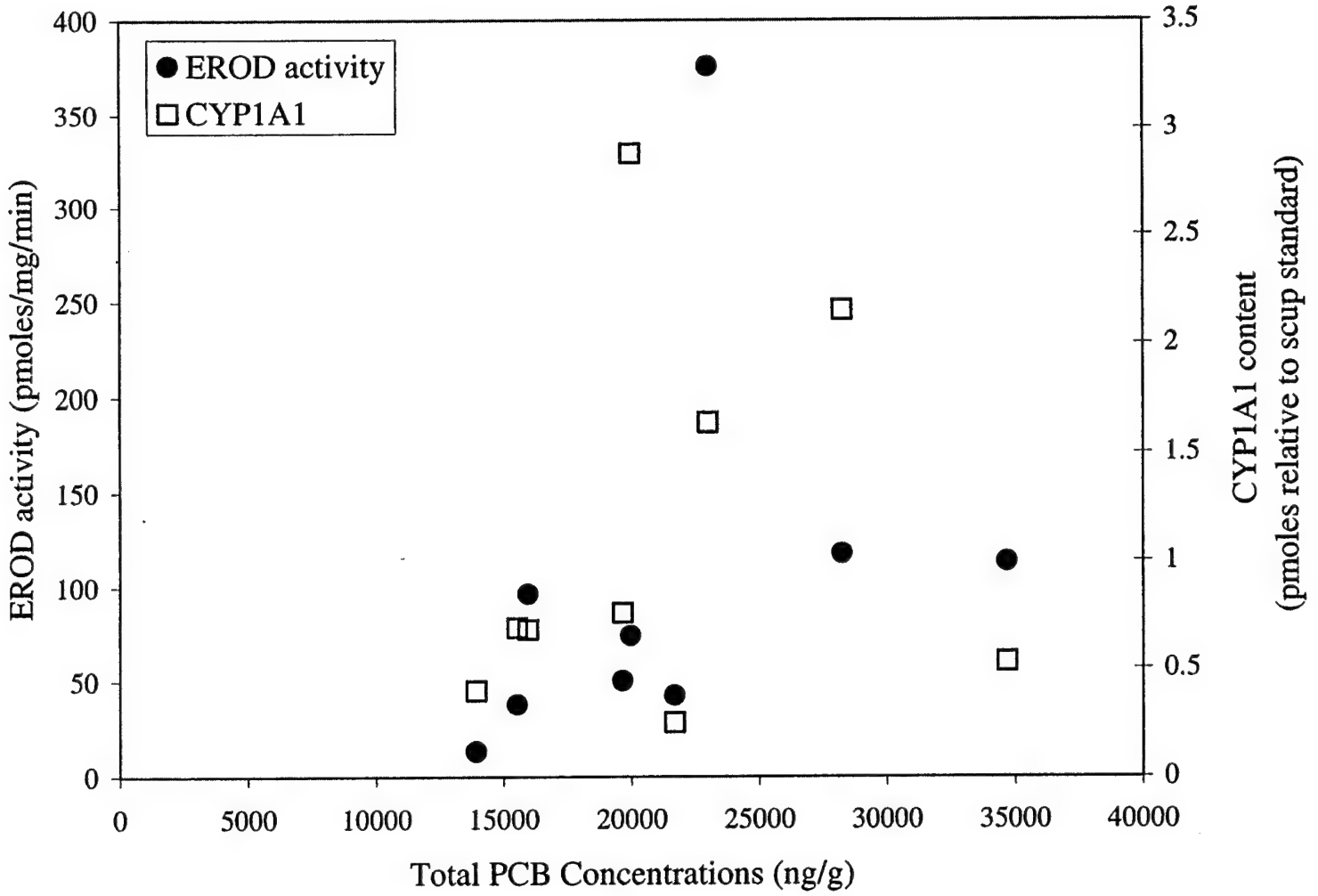
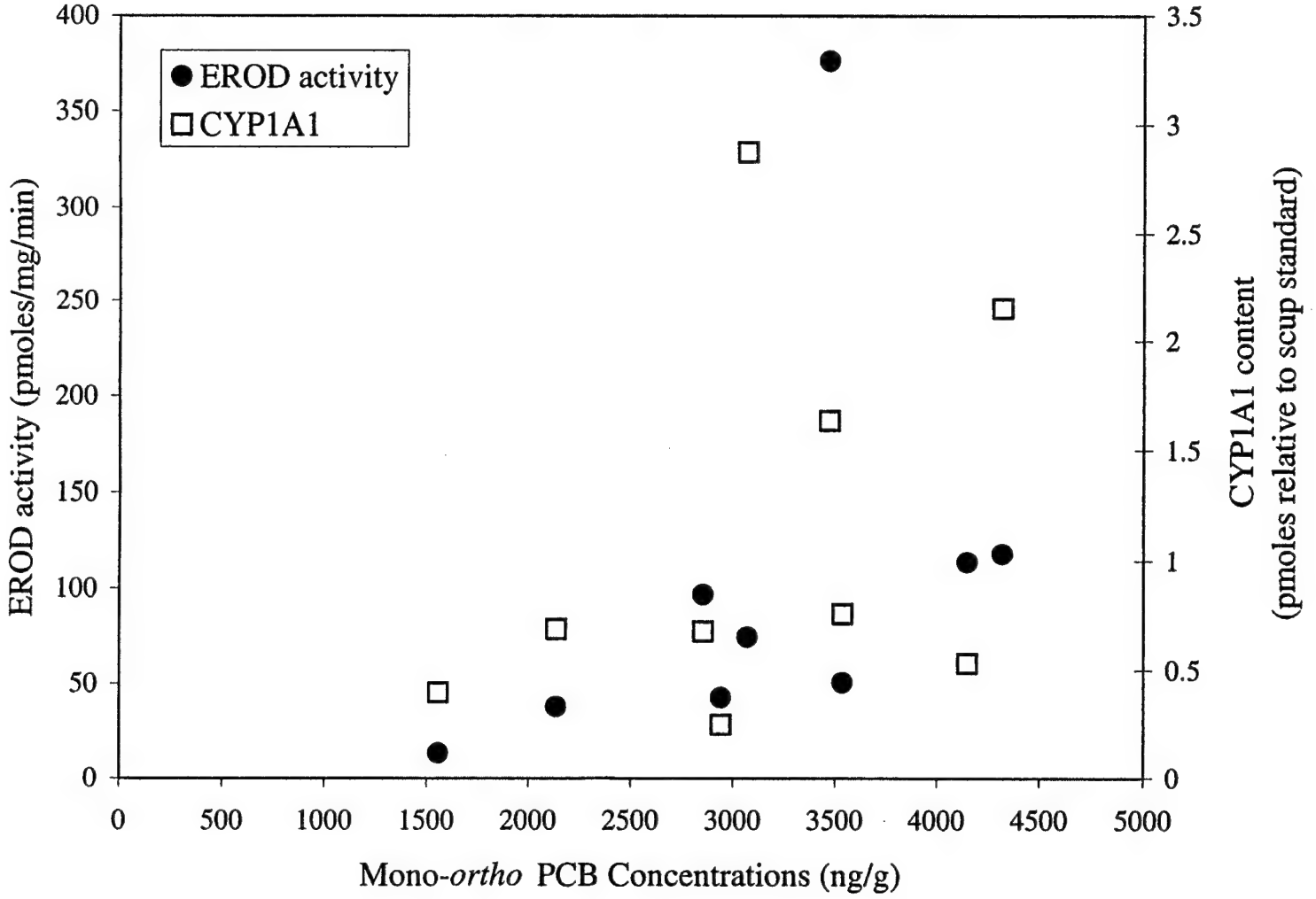

EROD activity
CYP1A1
EROD activity (pmoles/mg/min)
CYP1A1 content
(pmoles relative to scup standard)
Total PCB Concentrations (ng/g)
0
50
100
150
200
250
300
350
400
0
0.5
1
1.5
2
2.5
3
3.5
0
5000
10000
15000
20000
25000
30000
35000
40000

Figure 9. Liver ethoxyresorufin-O-deethylase activity and CYP1A1 content versus the Σmono-*ortho* PCB concentrations in blubber of white-sided dolphin. PCB concentrations are the sum of 16 PCB congeners measured in this study on a wet weight basis (see materials and methods for details).


EROD activity
CYP1A1
EROD activity (pmoles/mg/min)
CYP1A1 content
(pmoles relative to scup standard)
Mono-*ortho* PCB Concentrations (ng/g)
0
50
100
150
200
250
300
350
400
0
0.5
1
1.5
2
2.5
3
3.5
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000

Chapter 4. Cytochrome P450 1A1 (CYP1A1) Expression in Integument Biopsies from a Coastal Bottlenose Dolphin Population

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Abstract

Cytochrome P450 1A1 (CYP1A1) is an enzyme that is induced by polycyclic aromatic hydrocarbons (PAHs) and planar halogenated aromatic hydrocarbons (PHAHs) such as non- and mono- *ortho* substituted polychlorinated biphenyls (PCBs). Integument (epidermis and dermis/blubber) biopsies were collected from 47 bottlenose dolphins from a resident population in Sarasota Bay, FL. Cell type-specific CYP1A1 levels were measured in integument (epidermis and dermis/blubber) to assess the impacts of life-history parameters and contaminants concentrations on the expression of this protein. CYP1A1 expression was seen in vascular endothelial cells, vascular smooth muscle, and nerve cells in the dermis but not in epithelial cells, connective tissue, or adipocytes. Athough age and PCB concentrations were correlated, age, length and weight were not related to CYP1A1 expression. Sex did not appear to affect CYP1A1 expression. The Σmono-*ortho* PCB concentrations were positively correlated with endothelial CYP1A1 expression, although this relationship was dependent on just two males with the highest concentrations. Regional variation of endothelial CYP1A1 expression in the dermis was seen in this study and indicates that lipid dynamics, lipid or fatty acid content, or nutritional status may be important factors for endothelial CYP1A1 expression in integument.

Introduction

Determining effects of contaminants in cetacean populations is difficult because experimental exposures are precluded. Yet, disease and mortality events have been associated with high exposure to organochlorine contaminants in some cetaceans (Aguilar and Borrell 1994; Kuehl *et al.* 1994; Kuiken *et al.* 1994; Lipscomb *et al.* 1994; Lahvis *et al.* 1995; Guitart *et al.* 1996; Birkun *et al.* 1999; Jepson *et al.* 1999; Martineau *et al.* 2002). As part of the International Whaling Commission's (IWC) Pollution 2000+ program, live bottlenose dolphins (*Tursiops truncatus*) were sampled to evaluate potential biomarkers including CYP1A1 expression, of organochlorine contaminant exposure and effects. Polychlorinated biphenyls (PCBs) were identified as the chemicals of interest for this program because of their widespread global distribution and the extensive

information on the effects of these compounds for a variety of mammals. Planar halogenated aromatic hydrocarbons (PHAHs) are known to adversely affect the immune system, development and reproduction in mammals at low doses (Birnbaum and Tuomisto 2000). PHAHs, including the non- and mono-*ortho* substituted PCBs, are known to induce cytochrome P450 1A enzymes in vertebrates through binding to the aryl hydrocarbon receptor (Whitlock 1999). The induction of CYP1A1 by planar PCBs, polychlorinated dibenzo-*p*-dioxins and dibenzofurans has been correlated to their toxicity in rodent species (Safe 1986; Safe 1987; Safe 1990). CYP1A1 expression in liver has been correlated to concentrations of mono- and non- *ortho* substituted PCBs in blubber of beluga whale (White *et al.* 1994). The measurement of CYP1A1 expression in cetaceans and correlations of CYP1A1 expression with exposure in multiple cetaceans would allow comparisons of the responsiveness of different species to PHAH and PAH induction of CYP1A1. This comparison should allow us to determine the sensitivity of cetacean species to PHAH toxicity and allow us to infer the likelihood of toxic effects in wild populations where contaminant concentrations are known.

Obtaining tissue samples from cetacean species has relied on dead stranded animals, native subsistence hunts, or integument biopsies of free-ranging animals. Blubber collected from biopsy of wild cetaceans has been used for studies of exposure to organochlorine contaminants, including PCBs (Gauthier *et al.* 1997a; Gauthier *et al.* 1997b; Ross *et al.* 2000). Biopsy offers the best opportunity to sample from healthy animals over a wide range of ages and both sexes. CYP1A1 has been measured in several cell types present in cetacean integument biopsies from seventeen species (Miller *et al.* Submitted) and CYP1A activity has been correlated to organochlorine concentrations in striped dolphin and fin whale biopsies from the Mediterranean Sea (Fossi *et al.* 1992). Furthermore, a recent study in otters showed CYP1A1 induction in integument biopsies after exposure to crude oil (Ben-David *et al.* 2001). These studies demonstrate that CYP1A1 is present, measurable, and induced by exposure to typical CYP1A inducers in integument of marine mammals.

The Sarasota Bay bottlenose dolphin community provided opportunities to sample individual dolphins, with extensive background information on life history, reproductive

histories, ranging patterns, and health (monitored for four successive generations). The Sarasota-based research program has been developing methods for assessing the population status and health of coastal bottlenose dolphins, not only to monitor the risks to the populations themselves, but also to assess their use as sentinels of the health of marine ecosystems (Wells *et al.* In Press). Research on the resident Sarasota Bay dolphin community has been ongoing since 1970, and about 140 identifiable individuals, mostly of known gender, age, and genetic relationships, are currently monitored (Irvine and Wells 1972; Irvine *et al.* 1981; Scott *et al.* 1990; Wells and Scott 1990; Wells 2003). For this study, integument biopsy samples were collected from Sarasota Bay dolphins to determine CYP1A1 expression and concentrations of a variety of organochlorine contaminants, including PCBs. These samples are a representative cross-section of the resident dolphin community and facilitate a detailed analysis of the relationship between contaminant concentrations, age and gender on CYP1A1 expression in integument biopsies.

Materials and Methods

Integument samples (epidermis and underlying dermis or blubber) were collected from temporarily captured, free-ranging bottlenose dolphin (*Tursiops truncatus*) in Sarasota, Florida as part of an ongoing health and population assessment. This long-term monitoring program collects data on photographic identification, age, sex, reproductive events, and genetic relationships within the population. Captures took place in June of 1999, 2000, and 2001. All biopsies were taken from the same site on the dorso-lateral aspect and were the full depth of the integument that includes the epidermis and underlying dermis (i.e. skin and blubber). Lidocaine was applied in an L-block to the biopsy site prior to sampling. At least five minutes after the application of anaesthesia, a four by two cm wedge of integument was taken with a sterile scalpel. The removal site was packed with anti-coagulant until the time of release and after the bleeding was stopped. Biopsied animals have been re-examined, up to several years post-sampling, and there have never been any complications in the sampled animals (R. Wells, pers.

comm.). Matched samples of blubber were placed on ice and stored at -20°C. Integument was fixed in 10% neutral buffered formalin and stored at room temperature.

Immunohistochemical Analysis of CYP1A1 Expression

CYP1A1 expression was examined using immunohistochemistry, as previously described for fish (Smolowitz *et al.* 1991). Briefly, the formalin-fixed samples were embedded in paraffin and sectioned at 5μm. Sections were deparaffinated, hydrated and stained immunohistochemically using a peroxidase anti-peroxidase detection system (Signet Laboratories, Dedham, MA) with the monoclonal antibody 1-12-3 to scup (*Stenotomus chrysops*) CYP1A as the primary antibody. This antibody recognizes an epitope specific to CYP1A1 in mammals and detects CYP1A in taxonomically diverse vertebrates including cetaceans (Stegeman and Hahn 1994). Serial sections were labeled with the non-specific antibody MOPC31 (Sigma, St. Louis MO). Amino-9-ethylcarbazole (AEC) was used as the chromogenic substrate for visualization of CYP1A1. Nuclei were made visible by hematoxylin counterstaining. Stained sections were evaluated under light microscopy for stain occurrence (scale of 0-3) and stain intensity (scale of 0-5) in each cell type. CYP1A1 expression was calculated as the product of the stain occurrence and intensity to generate a semi-quantitative index (scale of 0-15). A linear relationship between this staining index and CYP1A protein content detected by immunoblot was shown previously (Woodin *et al.* 1997).

Blubber Contaminant Concentrations

Blubber samples weighing about 3g were ground with anhydrous sodium sulfate and extracted with n-hexane (residue-free quality) in a Soxhlet apparatus for 5 h. The solution obtained was concentrated to 40 ml and 10 ml were used to determine the quantity of extractable fat per gram of blubber. The remainder was mixed with sulfuric acid for lipid clean up and centrifuged for five minutes to aid separation of the hydrolyzed lipid from the solvent extract.

Chromatographic analysis was carried out on a Hewlett-Packard 5890-II G.C., equipped with an electron capture detector (ECD) at 350 °C. A fused silica capillary column (length 60 m, 0.25 mm ID) coated with SPB-1 was used as the stationary phase (0.25 um film thickness). The splitless technique was used to inject 1µl of the purified

extract. Pure nitrogen at a flow rate of 1 ml min^{-1} was used as a carrier gas. Temperature was programmed as follows: injection at 40 °C for one minute and increased to 170 °C at a rate of 25 °C$minute^{-1}$; one minute constant, to 250 °C at a rate of 2 °C $minute^{-1}$; and then to 280 °C, at 5 °C $minute^{-1}$.

A preliminary screening of the samples revealed that heptachlor was not present in the tissues analyzed. Therefore, this compound (0.1 mg kg^{-1}) was used as an internal standard. The samples were analyzed for the following compounds: HCB, p,p'-DDE, p,p'-DDD, o,p'-DDT, p,p'-DDT and polychlorinated biphenyls (PCBs). Concentrations were expressed in mg kg^{-1} lipid weight basis. Blanks of pure n-hexane were daily run to ensure the purity of the system. Recoveries of all the organochlorine compounds were calculated by adding known quantities of standard to 12 homogenate replicates of the same sample; they ranged 82 - 101%. The laboratory (A. Aguilar and A. Borrell, University of Barcelona, Spain) participated with satisfactory results in interlaboratory calibration exercises for organochlorines in biota organized by Quasimeme (1998) and NIST/NOAA (2000).

Total PCB concentration (tPCB) was calculated as the sum of 20 individual peaks (IUPAC# 95, 101, 110+136, 151, 135+144, 149, 153, 141, 138, 187,183+128, 174, 177, 171+202, 180, 170, 201, 196+203, 195, 194). Σmono-*ortho* PCB concentration was calculated as the sum of 4 individual peaks (IUPAC# 28, 105, 188, 156). Total DDT concentration (tDDT) was calculated as the sum of the four DDT compounds.

Statistical Analyses

All statistics were performed using Statistica 5.5 (Statsoft Inc. Tulsa, OK). Differences between males and females, for each CYP1A1 score and differences between cell types (both males and females) were detected using a T-test with $p<0.05$. Contaminant concentrations that were below the detection limit (0.001 mg kg^{-1}) were set at the detection limit for inclusion in statistical analyses. Data were ln transformed for correlations between age and PCB concentrations.

Results

Integument samples were collected from 59 known bottlenose dolphin (*Tursiops truncatus*; 23 males, 33 females, 3 unknown sex) from a resident population in Sarasota Bay, Florida. Animals were biopsied in the summers of 1999, 2000, and 2001, during temporary capture events as part of a long-term population and health assessment study. Matched blubber samples were taken from 47 animals for determination of contaminant concentrations. Males and females ranged in age from 2 – 43 and 2 – 50 years, respectively. The ages of the animals biopsied were evenly distributed. In males, which reach sexual maturity after 10 years, 45% of samples were from animals below the age of sexual maturity. In females, which reach sexual maturity as young as 5 years, 28% of samples were from animals below the age of sexual maturity. Males and females reach sexual maturity at 10 and 5 years of age, respectively. Ages were known for 80% of the animals sampled. Length and weight were correlated in both males ($r=0.98$, $p<0.05$) and females ($r=0.95$, $p<0.05$); however, age was only correlated to length ($r=0.93$, $p<0.05$) and weight ($r=0.93$, $p<0.05$) in males.

CYP1A1 expression was seen in endothelial cells of the arterial system and capillaries, vascular smooth muscle, and nerve cells (Figure 1). The nerve cell staining may include some fibroblasts, which are difficult to distinguish with this technique. CYP1A1 expression was not seen in epithelial cells, connective tissue, adipocytes and the perineurium, which is the dense connective tissue that surrounds the nerve bundle. The levels of CYP1A1 expression were not significantly different between males and females for any cell type (data not shown), and therefore, males and females were combined for subsequent analyses. CYP1A1 expression was lowest in nerve cells and highest in endothelial cells (Figure 2) and was statistically different between each cell type. Endothelial CYP1A1 expression was significantly greater than the levels found in both vascular smooth muscle and peripheral nerves.

Endothelial CYP1A1 staining showed regional variation within the dermis. Some cetaceans, including the bottlenose dolphin, have predominantly connective tissue and few or no adipocytes near the epidermal/dermal interface (Sokolov 1960). In the upper dermal region, endothelial CYP1A1 expression was significantly lower than in the rest of

the dermis where adipocytes are the dominant cell type (Figure 2). The upper dermis corresponds, in part, to the region often referred to as the outer blubber in many studies of lipid and fatty acid content in cetaceans.

CYP1A1 expression in endothelial cells from the lower dermis was correlated with CYP1A1 expression in both nerve cells ($r=0.62$, $p<0.05$) and vascular smooth muscle ($r=0.57$, $p<0.05$; Table 1). In contrast, CYP1A1 expression in endothelial cells from the upper dermis was not correlated to CYP1A1 expression in any other cell type (Table 1). CYP1A1 expression was correlated between smooth muscle and nerve cells ($r=0.72$, $p<0.05$) (Table 1). The life history parameters of age, length, and weight did not appear to have a strong influence on CYP1A1 expression as age, length, and weight were not significantly correlated with CYP1A1 levels in any cell type, regardless of whether sex was used as a grouping factor (data not shown).

Blubber was analyzed for several contaminants including polychlorinated biphenyls and the chlorinated pesticides α-, β-, and γ- hexachlorocyclohexane (HCH), hexachlorobenzene (HCB), aldrin and o,p- and p,p- dichlorodiphenyltrichloroethane (DDT) and their metabolites (o,p- and p,p- DDE and TDE). PCB congeners analyzed were mono-*ortho* and *ortho* substituted only (see materials and methods for list of specific congeners). A detailed analysis of contaminant concentrations will not be included in this paper. Total PCB and Σmono-*ortho* PCBs were calculated as the sum of all congener or mono-*ortho* congener (CB28, CB118, CB105 and CB156) concentrations. Contaminant concentrations commonly were cross-correlated with each other. Total PCBs were highly correlated to total DDTs ($r=0.99$, $p<0.05$), aldrin ($r=0.81$, $p<0.05$) and Σmono-*ortho* PCBs ($r=0.86$, $p<0.05$). The correlation between total PCBs and Σmono-*ortho* PCBs was less because of poor correlation between total PCBs and CB105 and CB28. Weaker correlations exist for total PCBs and all other pesticides except γ-HCH. Age – contaminant relationships were examined for total PCBs only. Total PCBs were linearly related to age for both males ($r^2=0.57$, $p<0.05$) and females ($r^2=0.45$, $p<0.05$) when both age and total PCBs were ln transformed (Figure 3).

CYP1A1 expression in vascular smooth muscle and nerve cells did not correlate with any contaminant concentration in the blubber when both males and females were included in the analysis. In females only, there was a correlation between CYP1A1 expression in vascular smooth muscle and β-HCH ($r=0.45$, $p<0.05$). Contaminants included in these analyses were HCB, α- β- and γ- HCH, ΣDDTs, total PCBs, and Σmono-*ortho* PCBs. CYP1A1 expression in endothelial cells from the upper dermis correlated with γ- HCH ($r=0.32$, $p<0.05$), but this relationship did not remain when animals were grouped by sex.

CYP1A1 expression endothelial cells from the lower dermis was correlated with total DDT, total PCBs, and Σmono-*ortho* PCB concentrations (Table 2). When animals were grouped by sex, a relationship between endothelial CYP1A1 in the lower dermis and Σmono-*ortho* PCBs was seen in males only (Table 2 and Figure 4). This relationship depended heavily upon 2 animals with very high Σmono-*ortho* PCB concentrations (Figure 4). The Σmono-*ortho* PCB concentrations were three to 4 standard deviations above the mean concentration for the male dolphins. The total PCBs concentrations were within one standard deviation of the mean for one dolphin (dolphin 106) but were 6 standard deviations above the mean for the other dolphin (dolphin 26). In spite of such different PCB concentrations, the CYP1A1 levels in these animals were within two standard deviations above the mean levels for the other dolphins.

The relative percent of each mono-*ortho* PCB was plotted for each male with all other *ortho*-substituted PCBs as reference (Figure 5). Dolphin 26 had a very similar PCB distribution (ie. relative contribution of each mono-*ortho* PCB congener to the total PCB concentration) as the other dolphins (Figure 5), in spite of having such a large difference in total PCB concentrations. Dolphin 106 had a very different PCB profile, with a much larger contribution from CB105, than the other dolphins (Figure 5). Dolphin 26 was the oldest male dolphin sampled, but dolphin 106, which has the highest concentrations of Σmono-*ortho* PCBs, is younger than 7 other male dolphins. If these two individuals are removed from the analysis, there is no correlation between Σmono-*ortho* PCBs and

CYP1A1 expression in endothelial cells (lower dermis, data not shown), indicating that the correlation is heavily dependant on these two samples.

Discussion

Previous work has demonstrated that CYP1A1 is expressed in several cell types present in cetacean integument and that this expression varies both within species, reflecting geographic differences, and between species (Miller *et al.* Submitted). The usefulness of measuring CYP1A1 as a biomarker of exposure in integument of free-ranging cetaceans will depend on our ability to demonstrate a dose-response relationship between CYP1A1 and contaminants. We will also need to remove or account for variables that may influence CYP1A1 expression, such as age, sex, and reproductive status because, for most populations of cetaceans, these factors would be unknown. The collection of biopsies from free-ranging animals does not usually involve temporary capture of the animal and collection of life-history data. The study of CYP1A1 expression in biopsies taken from such a well-studied population provides a rare opportunity to examine the influence of life-history parameters on CYP1A1 expression. Biopsy samples taken without capture of the animal are taken from different sites on the body, are from the superficial layers of the dermis, and penetration into the dermis varies. In this study, all samples were taken from the same site and were the full depth of the dermis. Sampling temporarily restrained animals allows for the collection of samples in a way that removes extraneous variability in the dataset.

Cell Types that Express CYP1A1 in Bottlenose Dolphin Integument

CYP1A1 expression was seen in vascular endothelial cells, vascular smooth muscle, and nerve cells but not in epidermal cells, adipocytes, and connective tissues. This is similar to results obtained in many other cetacean species, including bottlenose dolphin (Miller *et al.* Submitted). CYP1A1 expression has been seen in fibroblasts, a cell type of the connective tissue, from some cetaceans (Miller *et al.* Submitted) but was not seen in this study or in another study of bottlenose dolphin (J. Wilson, unpublished data, see appendix 2). The predominant cell type that expresses CYP1A1 in cetacean

integument is vascular endothelium (Miller *et al.* Submitted). In this study, also the highest CYP1A1 expression levels were seen in endothelium.

Endothelial CYP1A1 expression varied within the dermis. The upper dermis, an area characterized by connective tissue and few adipocytes, had lower levels of CYP1A1 expression than in the lower dermis, an area that is predominately adipocytes. Both common dolphin and bottlenose dolphin biopsies have regional differences in vascular endothelial CYP1A1 from the dermis (J. Wilson, unpublished data and Appendix 2), but this has not been seen in all cetacean species examined (C. Miller, pers. comm.). Certainly, the depth of the biopsy may be an issue in other species examined to date, particularly large whales, where the biopsy sample is typically a small proportion of the dermis (C. Miller, pers. comm.).

The reasons for regional differences in CYP1A1 expression in the dermis are unknown; differences in regional blood flow/perfusion and/or lipid dynamics may be involved. Interestingly, studies on lipid content and fatty acid analyses show that the outer blubber is structural and inner blubber is the active site for nutritive energy stores in cetaceans (Ackman *et al.* 1965; Ackman *et al.* 1971; Lockyer 1984; Aguilar and Borrell 1990; Koopman *et al.* 1996; Koopman *et al.* 2002). Although the outer blubber includes the region of the dermis (upper dermis) with lower endothelial CYP1A1 expression, the upper and lower dermis do not directly correspond to the inner and outer blubber of the aforementioned studies. The overlap of these regions still suggests that lipid content, fatty acid content and lipid dynamics may be important factors for endothelial CYP1A1 expression in cetacean integument. The nutrition status and reproductive status, particularly for females because of the energy demands of pregnancy and lactation, may have a major influence on the expression of CYP1A1 in endothelial cells of the lower dermis. Whether contaminant concentrations vary within the dermis is not yet clear. Contaminant concentrations in minke and blue whales, when expressed on a lipid basis, do not vary (Gauthier *et al.* 1997a), but concentrations in fin and sei whales were higher in the inner blubber, even when expressed on a lipid basis (Aguilar and Borrell 1991). Therefore, it is possible that differences in contaminant concentrations may account for the regional differences in CYP1A1 expression.

The Influence of Life-History Parameters on Contaminant Concentrations

Persistent contaminants such as PCBs accumulate over time and should increase with age. As expected, PCB concentrations are significantly correlated with age in these animals (Figure 3). This relationship between lipophilic persistent contaminant concentrations and age has been shown in several odontocetes including beluga whale (Martineau *et al.* 1994), pilot whale (Tanabe *et al.* 1987; Tilbury *et al.* 1999), killer whale (Ross *et al.* 2000), and harbour porpoise (Westgate *et al.* 1997; Westgate and Tolley 1999), as well as in the mysticete bowhead and right whale (Weisbrod *et al.* 2000; Hoekstra *et al.* 2002). As in this study, the concentration of PCBs increased with age in males but the relationship between contaminant concentrations and age in females appears to differ, depending on the species. In all species, except the beluga whale (Martineau *et al.* 1994), older reproductive females have lower blubber contaminant concentrations than younger non-reproductive females (Westgate *et al.* 1997; Tilbury *et al.* 1999; Ross *et al.* 2000; Weisbrod *et al.* 2000; Ylitalo *et al.* 2001). In several studies, contaminant concentrations increase until the age of first reproduction and then decline (Ross *et al.* 2000; Weisbrod *et al.* 2000). In some species, contaminant concentrations increase after senenscence (Tanabe *et al.* 1987; Ross *et al.* 2000; Weisbrod *et al.* 2000). In the dolphins in this study, females appear to have a continuous decline in contaminants, including PCBs, during their lifespan; there is no accumulation in the period before sexual maturity (age 5). Female harbour porpoise have nearly constant contaminant concentrations until the age of reproduction (4 years); after sexual maturity, concentrations decline (Westgate *et al.* 1997). In both the harbour porpoise (Westgate *et al.* 1997) and bottlenose dolphin included in this study, a sampling bias cannot explain the results, as samples were obtained from animals with ages covering most of the lifespan. The youngest bottlenose dolphins in this study (age 2 years) represent animals that have just weaned and separated from their mothers. The reasons for a continuous decrease in contaminant concentrations over time is unclear; although at later ages, the continuing decline is because, similar to harbour porpoise, bottlenose dolphin are reproductively active throughout their lifetime. Females in the Sarasota population have successfully reproduced at age 45, an age near the limit of their lifespan (Wells and Scott 1999).

The Influence of Life-History Parameters on CYP1A1 Expression

CYP1A1 expression does not appear to be related to the age, length, or weight of the animal regardless of whether sex is used as a grouping factor. This is surprising considering that age and PCB concentrations are related by age. However, the age-contaminant relationship is complicated, particularly for females where the data needed to be ln transformed to linearize it. Considering that CYP1A1 expression also has regional variation in the dermis and contaminant concentrations were measured in whole dermis, variability in the relationship between contaminant concentration and both age and CYP1A1 levels likely obscured any relationship between age and CYP1A1 expression. Ultimately, this data suggests that age would merely be cross-correlated and is not an important factor in CYP1A1 expression. This is important information for the interpretation of biopsies from offshore animals or coastal populations that lack the long-term monitoring data of the Sarasota FL bottlenose dolphin population, and implies that interpretation of CYP1A1 expression may be made without knowledge of these parameters, at least in this species.

The Influence of Contaminants on CYP1A1 Expression

Since CYP1A1 is induced by PAHs and PHAHs including planar PCBs, we would expect that CYP1A1 expression might correlate with concentrations of either total or Σmono-*ortho* PCBs. PAH concentrations were not measured in this study and their contribution to CYP1A1 expression remains unaddressed. In Arctic beluga, CYP1A1 expression in liver was correlated with Σmono-*ortho* PCBs in blubber, and this correlation was not dependant on sex (White *et al.* 1994). Otters, that were experimentally fed crude oil that contained PAHs capable of inducing CYP1A1, had a dose-dependant increase in CYP1A1 expression in integument (Ben-David *et al.* 2001). These studies demonstrate that CYP1A1 expression and the concentration of CYP1A1 inducers should be related. Studies of the relationship between contaminant concentrations and benzo(a)pyrene monooxygenase (BPMO) activity, which is catalyzed by CYP1A1, in cetacean species from the Mediterranean Sea have shown weak relationships with DDTs and PCBs (r^2<0.52, Fossi *et al.* 1992; Marsili *et al.* 1998). In

those studies, they did not test for associations between the non- and/or mono- *ortho* PCBs and BPMO activity.

In this study, CYP1A1 expression in endothelial cells of the lower dermis was correlated to the Σmono-*ortho* PCBs in males but not females. However, all female dolphins and the majority of male dolphins had Σmono-*ortho* PCBs less than 15 mg kg^{-1} in their blubber, and only 2 males had concentrations above this amount. Since the correlation between CYP1A1 and Σmono-*ortho* PCBs is present only with these two animals included in the analysis, CYP1A1 expression appears to be difficult to predict in a range of contaminant concentrations of less than 15 mg kg^{-1}. With the two animals (Dolphin 26 and 106) with higher Σmono-*ortho* PCBs concentrations, the range of contaminant concentrations more than tripled to a range of nearly 50 mg kg^{-1} (Figure 4).

For Dolphin 106, CB105 accounted for over 30% of its total PCB concentrations, while this congener accounted for less than 3% of total PCBs in all other males (Figure 5). This individual male appears to have a very different PCB spectrum than the other animals in the study. Dolphin 26 appeared to have the same relative contribution of each PCB congener compared to the other male dolphins sampled (Figure 5). Adequate sampling of male dolphins with 20 – 50 mg kg^{-1} Σmono-*ortho* PCBs would be needed to confirm the strength of the correlation between contaminants and Σmono-*ortho* PCBs. Future work should focus on older male animals to obtain samples from animals with moderate to high contaminant concentrations for this population.

There was no correlation between CYP1A1 expression in the lower dermis and Σmono-*ortho* PCBs in female dolphins (Figure 4). However, this may simply reflect the lower contaminant concentrations in females and not a different response pattern between males and females. As stated above, there was no correlation between CYP1A1 expression and the Σmono-*ortho* PCBs in males when the analysis excluded the two males with the highest concentrations, concentrations that were more than 3 standard deviations above the mean. Furthermore, the inclusion of both males and females into the analysis did not remove the correlation between CYP1A1 and Σmono-*ortho* PCBs because most females clustered with males of similar contaminant concentrations (Figure

4). It appears that sex is not a key factor in the interpretation of CYP1A1 expression *per se*, but that contaminant concentration alone is sufficient to explain CYP1A1 expression. This interpretation would agree with a study of CYP1A1 expression in liver of beluga whales, where both males and females were included in a rather striking correlation between CYP1A1 and Σmono-*ortho* PCB concentrations in blubber (White *et al.* 1994). In the beluga study, the females had lower PCB concentrations and CYP1A1 expression, in essence making up the lower half of the range. The range of contaminant concentrations included in the analysis is clearly very important because when the range of Σmono-*ortho* PCB concentrations is less than 15 mg kg^{-1}, the correlation is lost. In contrast to females, males tend to accumulate PCBs with time. Thus, male dolphins offer the best opportunity to sample the breadth of contaminant exposures within a population.

Comparisons of CYP1A1 Expression Between Cetacean Species

The levels of CYP1A1 expression seen in Sarasota bottlenose dolphin are similar to those seen in many other odontocete species (Miller *et al.* Submitted). Mean CYP1A1 expression in endothelial cells is usually ≤8 (scale of 0-15), approximately the expression level seen in the lower dermis of bottlenose dolphin in this study. CYP1A1 expression in this coastal bottlenose population was similar to that reported in other coastal populations of this species (San Diego and the Gulf of Mexico) but higher than that reported from bottlenose dolphin from the Mediterranean Sea and offshore bottlenose dolphin from the Western Atlantic Ocean (Miller *et al.* Submitted). Contaminant concentrations were not measured in previous studies of CYP1A1 in biopsies from bottlenose dolphin (Miller *et al.* Submitted), although we would expect lower contaminant concentrations in the offshore bottlenose dolphins. The measurement of contaminant concentrations, particularly in those bottlenose dolphin samples for which there are CYP1A1 data, would be helpful for the comparison of these studies.

When compared to populations with known contaminant concentrations, CYP1A1 expression in integument suggests differences in sensitivity between species. Arctic beluga, with blubber PCB concentration of less than 5 mg kg^{-1} (Muir *et al.* 1996), express similar levels of CYP1A1 in endothelial cell as the Sarasota bottlenose dolphin (this

study and Miller *et al.* Submitted). Yet the male Sarasota bottlenose dolphin have blubber PCB concentrations that are on average 20 times higher than male Arctic beluga. This would suggest that the beluga whale is more sensitive than bottlenose dolphin to PHAH induction of CYP1A1. Similarly, killer whales from the Pacific Northwest have some of the highest PCB concentrations recorded for cetaceans (Ross *et al.* 2000), and yet CYP1A1 expression in animals from this same region is among the lowest seen to date (Miller *et al.* Submitted). It is possible that killer whales are comparatively unresponsive to PCB concentrations and that the higher exposures are still not sufficient to induce CYP1A1 in this species.

Conclusions

The usefulness of CYP1A1 expression in integument as a biomarker of PCB exposure and toxic effects is not yet clear. However, the results of this study indicate that as with liver CYP1A1 expression (White *et al.* 1994), endothelial CYP1A1 expression in integument is correlated with Σmono-*ortho* PCB concentrations. Additional samples from older males and measurements of PAH exposure in this bottlenose dolphin population would address how strong a relationship there is between integument CYP1A1 and PCB concentrations in this population. That this relationship has been found in two different tissue types and with different species is an indication that CYP1A1 expression may be widely applicable as a biomarker of exposure in cetacean species. The application of CYP1A1 measurements in populations with documented pathologies or disease related to PHAH exposure will help validate the use of CYP1A1 expression as a biomarker of PHAH effects. Certainly, an ongoing effort to understand the role of lipid dynamics, lipid and fatty acid content, nutritional, and reproductive status will be needed to understand the controlling factors of CYP1A1 expression in integument biopsy samples. The life history parameters of age, length, weight and sex do not appear to have a strong influence on CYP1A1 expression which will allow us to apply this technique to free ranging cetacean populations where this data is mostly lacking.

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Table 1. Correlations between cell-specific expression of cytochrome P4501A1 (CYP1A1) in integument of bottlenose dolphin. The correlation coefficient is shown above the diagonal and the significance is shown below (NS = not significant, * = $p<0.05$).

	Endothelial Cell-Upper Dermis	Endothelial Cell-Lower Dermis	Vascular Smooth Muscle	Nerve Cell
Endothelial Cell-Upper Dermis	-	0.22	0.29	0.09
Endothelial Cell-Lower Dermis	NS	-	0.57	0.62
Vascular Smooth Muscle	NS	*	-	0.72
Nerve Cell	NS	*	*	-

Table 2. Correlations between expression of cytochrome P4501A1 (CYP1A1) in endothelial cells of the lower dermis and contaminant concentrations in integument of bottlenose dolphin. Correlations were performed with all congeners of PCBs or DDTs, or with the Σmono-*ortho* PCBs only. The correlation coefficient is shown, and correlations with $p<0.05$ are marked (*).

	Total DDTs	Total PCBs	Σmono-*ortho* PCBs
Males only (N=22)	0.33	0.36	0.52 (*)
Females only (N=25)	-0.28	-0.25	-0.28
All Samples (N=47)	0.34 (*)	0.38 (*)	0.49 (*)

Figure 1. Cytochrome P4501A1 (CYP1A1) Expression in Integument Biopsies of Bottlenose Dolphin. CYP1A1 expression is shown in A) endothelial cells, B) vascular smooth muscle, and C) nerve cells. Panel D shows a serial section of the samples shown in panel B stained with the non-specific antibody, MOPC31. CYP1A1 expression was determined using immunohistochemistry. CYP1A1 expression is labeled red. Arrows (Panels A-C) identify cells with CYP1A1 expression, arrowheads (Panel D) identify endothelial cells and smooth muscle cells lacking CYP1A1 staining. All panels are at 400x magnification.

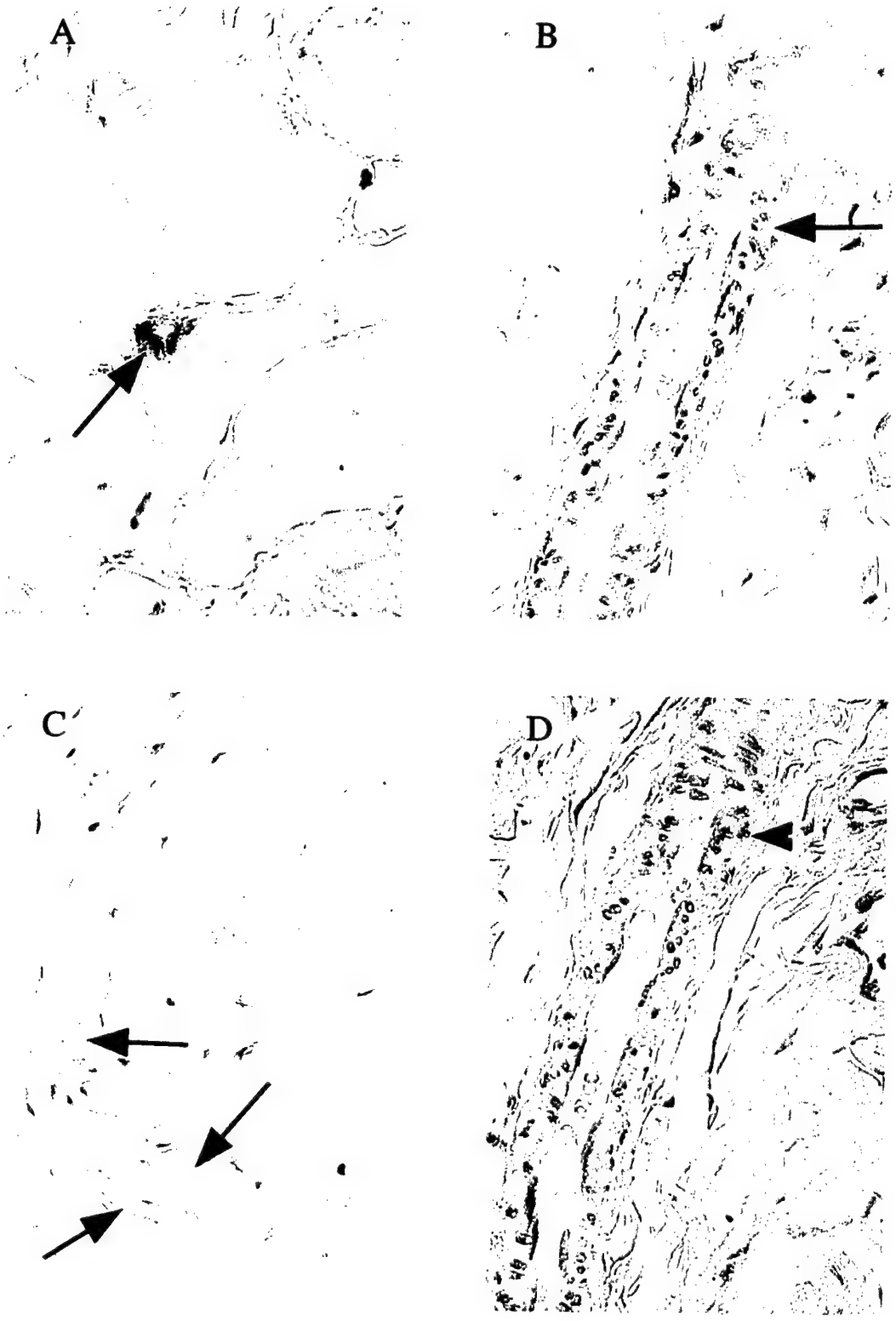
A
B
C
D

Figure 2. Mean Cytochrome P4501A1 (CYP1A1) Expression in Integument Biopsies from Bottlenose Dolphin. Only those cell types that were found to express CYP1A1 are shown. Endothelial cells were divided into two geographical regions in the biopsy, the upper and lower dermis (see text for details). Each bar represents a mean of all dolphins (male and female) included in this study (N=59), except for nerve cells (N=33) which were not present in every tissue slice examined. Those bars with different letters are significantly different ($p>0.05$) from each other.

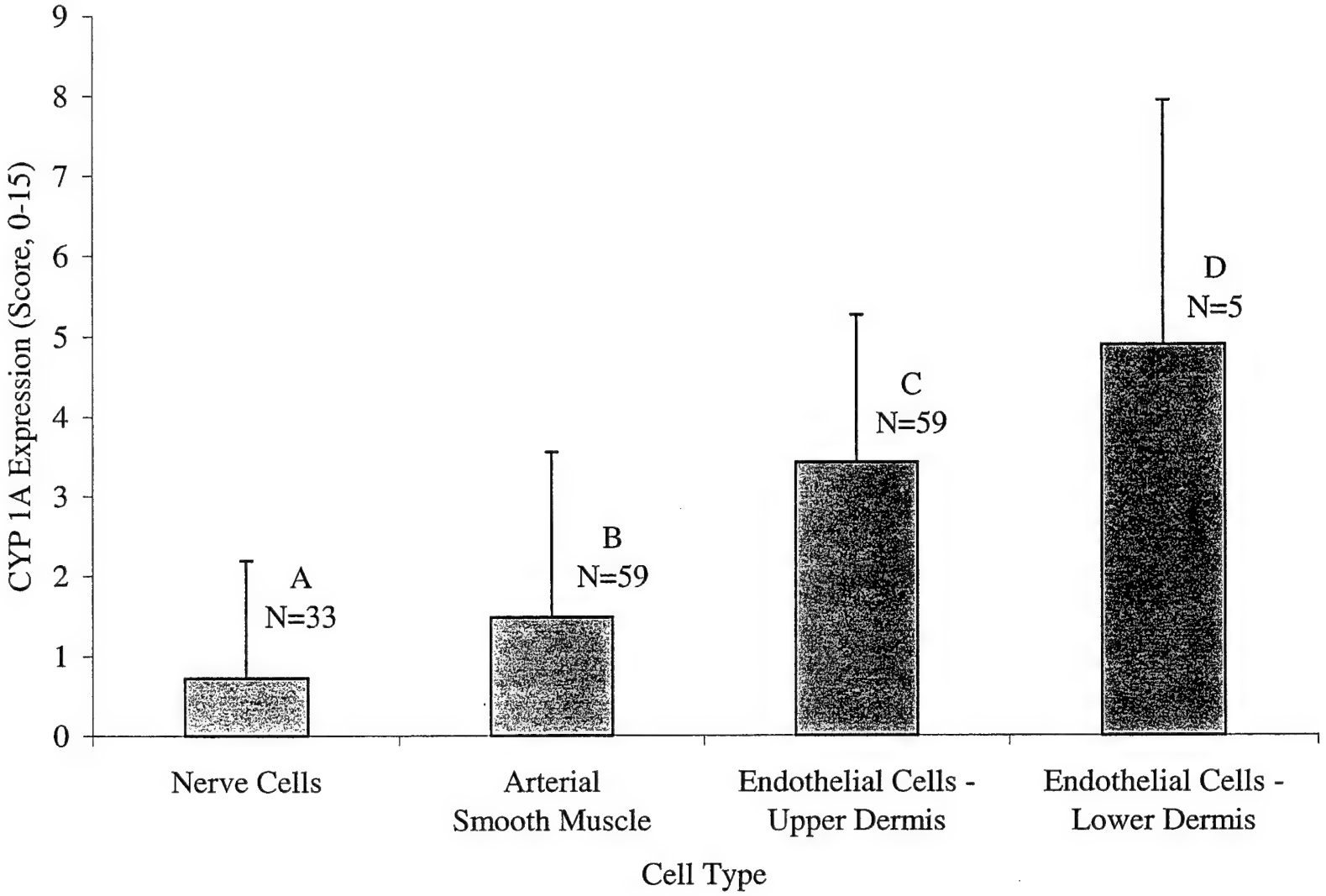
CYP 1A Expression (Score, 0-15)
9
8
7
6
5
4
3
2
1
0
A
N=33
B
N=59
C
N=59
D
N=5
Nerve Cells
Arterial Smooth Muscle
Endothelial Cells - Upper Dermis
Endothelial Cells - Lower Dermis
Cell Type

Figure 3. PCB Concentrations in Blubber Vary as a Function of Age in Bottlenose Dolphin. Regression of ln (total PCB concentrations in blubber) and ln age in male and female bottlenose dolphin. Regressions are significant ($p<0.05$) for both males and females. See materials and methods for complete listing of congeners included in total PCBs.

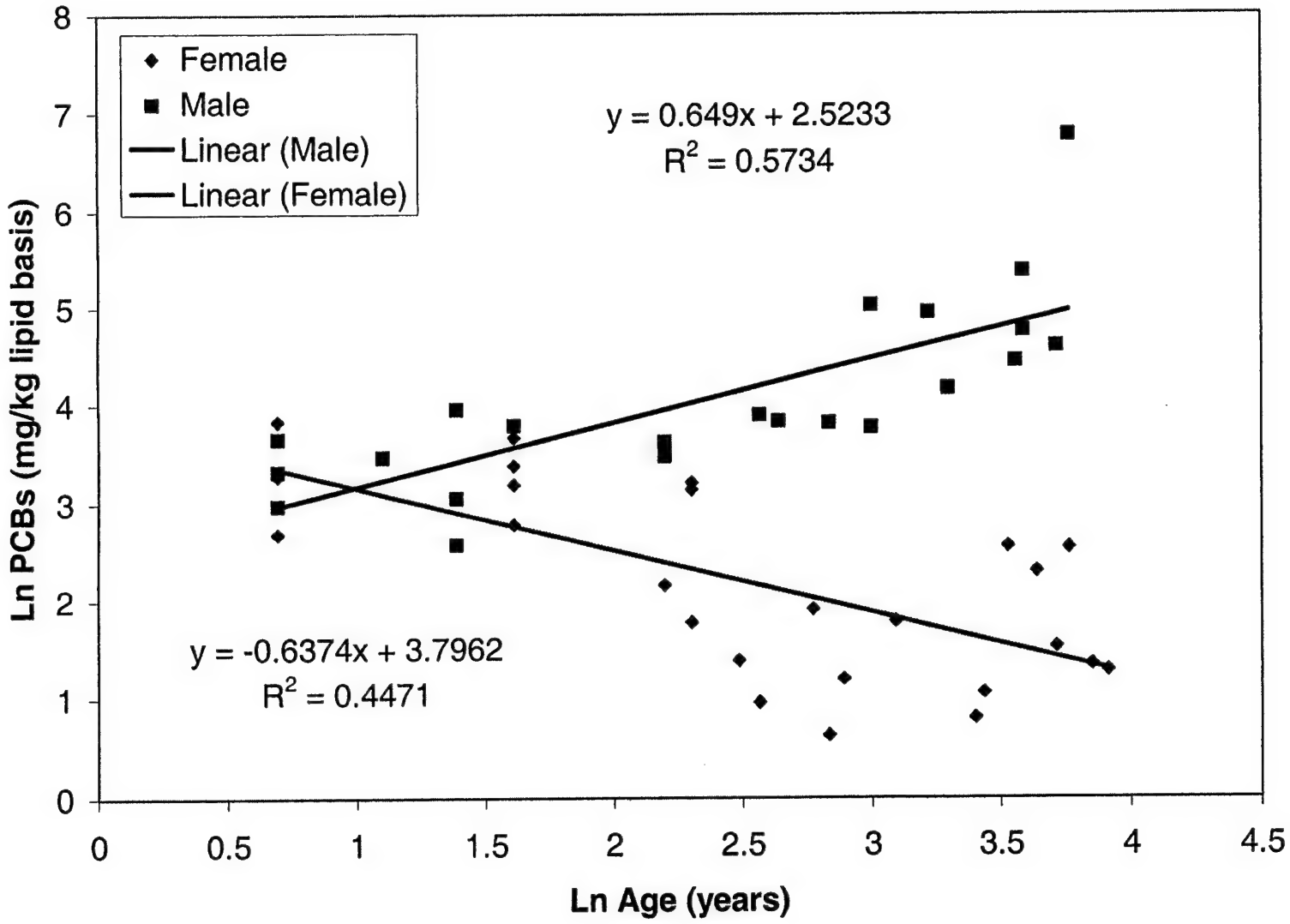
Female
Male
Linear (Male)
Linear (Female)
y = 0.649x + 2.5233
R2 = 0.5734
y = -0.6374x + 3.7962
R2 = 0.4471
Ln PCBs (mg/kg lipid basis)
Ln Age (years)
0
1
2
3
4
5
6
7
8
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

Figure 4. Correlation of Σmono-*ortho* PCBs and CYP1A1 Expression in Endothelial Cells from the Lower Dermis.
PCB concentrations are the sum of CB28, CB205, CB118 and CB156. Correlations are significant ($p<0.05$) when both males and females are included in the analysis and for males only. The correlation is not significant when restricted to females only.

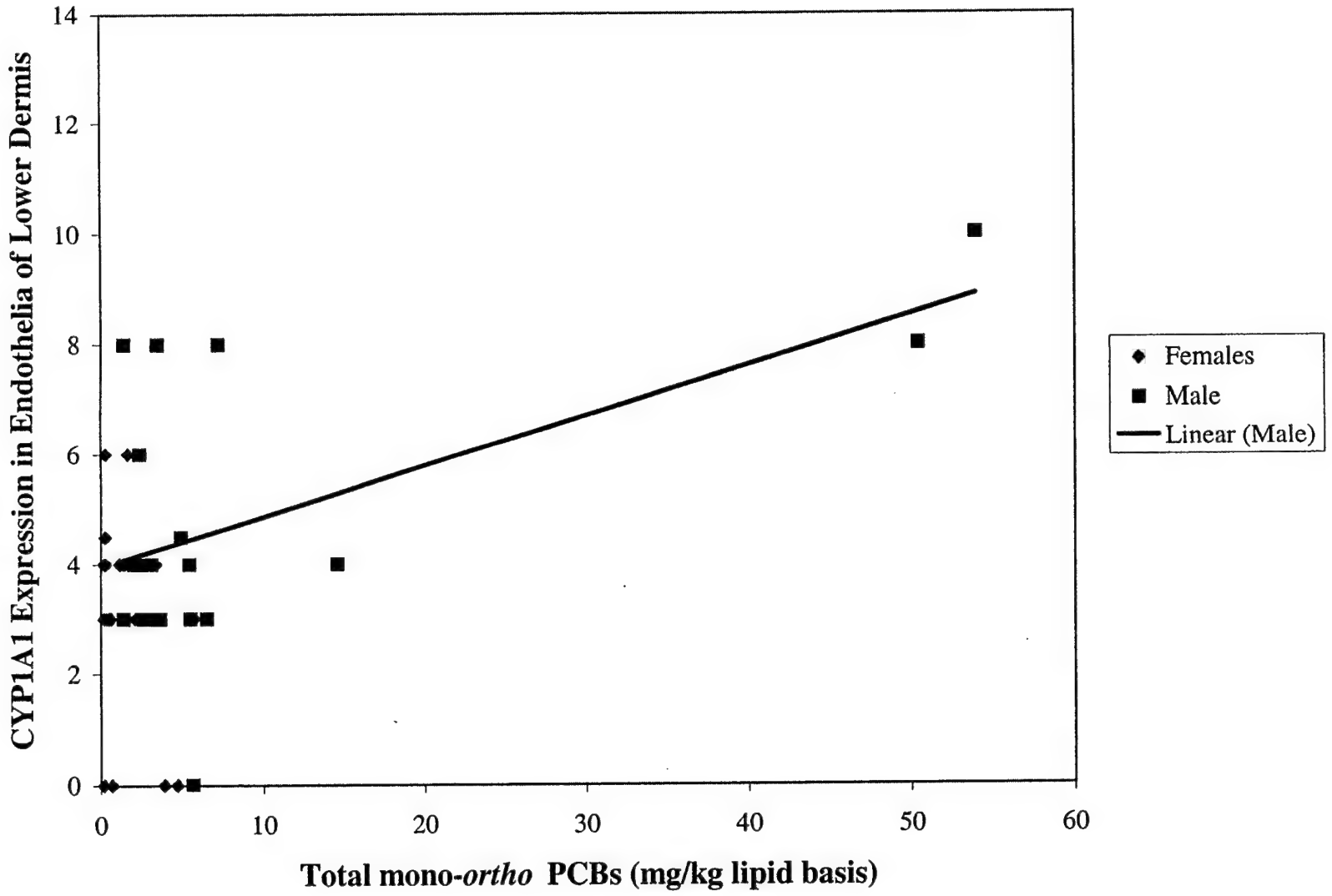
CYP1A1 Expression in Endothelia of Lower Dermis
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
Total mono-ortho PCBs (mg/kg lipid basis)
Females
Male
Linear (Male)

Figure 5. Relative Contribution of Each Mono-*ortho* PCB Congener to the Total PCB Concentration in Male Bottlenose Dolphin. Concentrations are expressed as a percent of the total PCB concentration for that animal. Each mono-*ortho* congener measured (CB28, CB205, CB118 and CB156) is shown relative to all other PCB congeners measured (see materials and methods). Dolphin 26 and 106 are the animals with the highest (>50 mg kg^{-1}) Σmono-*ortho* PCBs in this study.

Percent PCB Congeners
(of Total PCBs, mg/kg lipid basis)
100%
80%
60%
40%
20%
0%
Dolphin
6
14
26
28
32
48
58
66
92
106
118
138
146
148
174
178
182
196
198
212
214
216
All other congeners
CB156
CB105
CB118
CB28

Chapter 5. Characterization of a Cetacean Aromatase (CYP19) and the Phylogeny and Functional Conservation of Vertebrate Aromatase

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Abstract

Aromatase (cytochrome P450 19, CYP19, P450arom) is the enzyme responsible for the production of estrogens, hormones critical for development and reproduction. Aromatase was sequenced from a white-sided dolphin (*Lagenorhynchus acutus*) ovary and transiently transfected into HEK 293 cells. The K_ms for androstenedione and testosterone were 63.5 and 75 nM, respectively; values that are very similar to those reported for other mammalian aromatases. A Bayesian phylogenetic analysis of the vertebrate aromatases, including fish, amphibians, reptiles, birds and mammals, was performed on the amino acid sequences. Based on known species phylogeny, the cetacean aromatase showed an expected grouping with artiodactyls (cow, sheep, and goat). A functional divergence analysis showed a strong conservation across the entire aromatase protein and that the observed sequence divergence is functionally neutral.

Introduction

Aromatase (CYP19, P450arom) is the primary enzyme responsible for the generation of estrogens from their precursor androgens (Corbin *et al.* 1988). Aromatase expression is usually limited to gonads and brain within the vertebrates, although it is also expressed in placenta of primates and artiodactyls, and widely expression in humans (Simpson *et al.* 1994; Conley and Hinshelwood 2001a; Simpson *et al.* 2002). The expression of aromatase is critical for reproductive success. Mutations in *CYP19* have been linked to infertility in humans (Adashi and Hennebold 1999) and rodents (Hany *et al.* 1999). In humans, where aromatase is expressed in many internal organs, estrogen also appears to be important for vascular function, lipid and carbohydrate metabolism and bone mineralization (reviewed in Simpson *et al.* 2002). The extensive involvement of estrogen in both reproductive and non-reproductive functions in mammals is supported by recent studies in aromatase knockout mice (Fisher *et al.* 1998; Honda *et al.* 1998; Robertson *et al.* 1999; Agarwal *et al.* 2000; Jones *et al.* 2000; Britt *et al.* 2001; Jones *et al.* 2001; Toda *et al.* 2001; Murata *et al.* 2002).

In spite of its importance, the aromatase gene has been studied in relatively few mammals and only extensively in humans, where it is known to play a significant role in

pathophysiology, such as in breast cancer. The full coding sequence of the *CYP19* gene (which encodes aromatase) has been sequenced from only 11 mammalian species (goat, horse, cow, sheep, pig, human, marmoset, macaque, mouse, rat and rabbit). With the demonstration of 3 porcine aromatase genes (Choi *et al.* 1996; Corbin *et al.* 1999b; Graddy *et al.* 2000), but only a single human, bovine, and equine aromatase, aromatase is thought to be present in a single copy in most mammals (Conley *et al.* 2001a; Simpson *et al.* 2002). Cetaceans (whales and dolphins) are most closely related to ruminant artiodactyls such as the cow (Gatesy and O'Leary 2001). Sequencing and heterologous expression of novel aromatase sequences, particularly in the ungulate clade where multiple aromatase genes exist in at least one species, may enhance our understanding of the conservation and function of this important protein across the Mammalia.

Since many cetacean species were subject to intense hunting and their recovery is not yet certain, successful reproduction in these species is often critical for their survival. Studies that examine genes important for reproduction are needed in cetacean species. Experimental studies of marine mammals are precluded, which makes the understanding of the similarities and differences of reproductive systems between mammalian taxa critical. Effective extrapolation of information from model species requires a comparative approach. The *CYP19* gene and estradiol function appears to be strongly conserved across the Mammalia (Conley *et al.* 2001a). However, whether information on the function and control of estrogens in model organisms could be applicable to mammalian wildlife species is not clear. The objective of this research was to establish a molecular foundation for understanding estrogen production in cetaceans and to assess functional differences among aromatase, from both a catalytic and an evolutionary perspective. We cloned a full-length *CYP19* from a white-sided dolphin (*Lagenorhynchus acutus*) ovary and performed transient transfection experiments for *in vitro* characterization of this cetacean aromatase. We investigated aromatase diversification by performing a Bayesian phylogenetic analysis of 41 aromatase amino acid sequences that include mammalian, amphibian, reptile, bird, and fish forms. To examine the evolution of aromatase function, we combined phylogenetic analysis with statistical analysis of sequence divergence.

Materials and Methods

Tissue Preparation

Ovary of a white-sided dolphin (accession number MH-97-534) was collected from an animal that stranded on Cape Cod and was subsequently euthanized. Ovary was frozen in liquid nitrogen approximately 7 hours after death. Total RNA was isolated using Stat60 (Tel-test, Friendswood, TX) and poly A^+ mRNA was isolated from total RNA using a mini Oligo (dT) poly A^+ cellulose column spin kit (5'→3' Prime Inc., Boulder, CO).

Isolation of Dolphin CYP19 cDNA

Two overlapping fragments (702 and 830 bp, covering a total of 1332 bp) of the aromatase gene were amplified from total RNA using the GeneAmp RNA PCR kit (Applied Biosystem, Foster City, CA). Total ovary RNA (1.1ug) was reverse transcribed by random hexamers and oligo d(T). Amplification was performed in the same tube with degenerate mammalian primers (Table 1, internal set 1 and 2). PCR was initiated by melting at 94°C for 7 minutes followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 90 s, all concluded by an extension at 72°C for 10 min. Rapid amplification of cDNA ends (RACE) was performed to completely determine the coding region of the gene. Multiple 5' and 3' RACE products were generated using nested primers (Table 1) and poly A^+• mRNA with the Marathon RACE kit (Clontech, Palo Alto CA). RACE ready cDNA was generated using 0.81 μg poly A^+ RNA according to RACE kit instructions. In separate reaction mixtures, 0.2 ul of RACE ready cDNA, served as template with gene specific RACE primers, 3'RACE1, 3'RACE2, 5'RACE2 and 5'RACE3 (Table 1). The RACE reactions were carried out using a touchdown protocol: initial melting at 94°C for 30 s, followed by 5 cycles of 94°C for 5 s and 72°C for 3 min, 5 cycles of 94°C for 5 s and 70°C for 3 min, 25 cycles of 94°C for 5 s and 68°C for 3 min. Nested RACE was performed for the 5'RACE reactions using the nested RACE primer 5'RACE (Table 1). Nested RACE reactions were performed using a touchdown protocol as outlined above for RACE except that the final cycles at 94°C for 5 s and 68°C for 3 min were performed for 15 cycles only. The RT-PCR and RACE fragments were gel purified (Geneclean II, Bio 101 Systems, Vista CA), ligated into the pGEM-T Easy

vector (Promega, Madison WI), and transformed into competent JM109 *E. coli* cells. Colonies with inserts were cultured overnight and recombinant DNA was prepared for sequencing using a QiaPrep miniprep kit (Qiagen, Valencia CA). Clone sequences were generated using a cycle-sequence protocol (Epicentre Technologies, Madison, WI) with IR-labeled vector-primers (M13F and M13R) and resolved on a LI-COR 4200L sequencing apparatus (LI-COR Inc., Lincoln, NE). For the RT-PCR fragments, 4 clones from the 830 bp and 3 clones of the 702 bp fragments were sequenced in both forward and reverse direction. For the RACE reactions, 14 clones from the 3'RACE reactions (6 clones from 3'RACE1 and 8 clones from 3'RACE2 reactions) and 6 clones from the nested 5'RACE reactions were sequenced in both directions. Contig assemblies were generated with Sequencher (GeneCodes, Ann Arbor MI).

Primers

Degenerate mammalian *CYP19* PCR primers were designed based on a DNA alignment of full length coding sequences of *CYP19* from cow, horse, pig, mouse, rat, rabbit, and human. The GenBank accession numbers of these nucleotide sequences are listed in Table 2. The DNA alignment was generated with Clustal (Higgins 1994). PCR and RACE Primer sequences are listed in Table 1.

Phylogenetic and Functional Analyses

CYP19 amino acid sequences used for phylogenetic analyses were retrieved from GenBank by extensive BLAST searches and are listed in Table 2. Alignment and analysis were performed as described elsewhere (McArthur *et al.* 2003). In brief, sequence alignment was performed using ClustalX 1.81 (Thompson *et al.* 1997), with manual correction by eye. The stingray sequence was designated as the outgroup. Phylogenetic relationships of the CYP19 sequences were assessed using the computer program MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). MC^3 (metropolis coupled, markov chain, monte carlo) estimation of posterior probabilities was performed using noninformative prior probabilities, the JTT+I+Γ (Jones *et al.* 1992) substitution model with inclusion of unequal amino acid frequencies, and four incrementally heated Markov chains with different random starting trees. The Markov chains were run for 10,000,000 generations, with sampling of topologies every 100 generations. Posterior probabilities

of topologies, clades, and parameters were estimated from the sampled topologies after removal of MC^3 burn-in. A functional divergence analysis of the amino acid alignment in the context of the hypothesized phylogenetic tree was performed using the computer program DIVERGE (Gu and Vander Velden 2002). DIVERGE was used to test the null hypothesis of no changes in site-specific evolutionary rates among CYP19 sub-clades and to predict sites in the alignment having altered functional constraints. These sites may have been subject to divergent functional evolution. DIVERGE measures change in site-specific evolutionary rates using the coefficient of evolutionary functional divergence (θ), where $\theta = 0$ indicates no change and values approaching $\theta = 1$ reflect increasing functional divergence. Specifically, θ measures site-specific divergence from a homogenous gamma model of among-site rate variation.

Transient Transfections of Dolphin CYP19

The full-length coding region of dolphin *CYP19* was generated by RT-PCR using the primers fullcds_CYP19F and fullcds_CYP19R (Table 1). The sequence of the full-length fragment was verified and it was cloned into the pCI-neo mammalian expression vector (Promega, Madison WI). The vector, pCI-neo-dCYP19, and an empty vector, pCI-neo-control, were grown in JM109 *E. coli* cells and vector DNA was purified using the EndoFree Maxi Prep kit (Qiagen, Valencia CA). The pCI-neo-dCYP19 or the pCI-neo-control vectors were co-transfected into HEK 293 cells with the pRL-TK Renilla luciferase expression vector (Promega, Madison WI). HEK 293 cells were grown in Eagle's MEM (Sigma, St. Louis MO) with 1.5 g L^{-1} sodium bicarbonate, 10 mM sodium pyruvate, 10mM HEPES and 10% heat inactivated horse serum (Sigma, St. Louis MO).

Transient transfections were completed using lipofectamine 2000 reagent (Life Technologies, Carlsbad CA) diluted with vector DNA in serum free media according to manufacturer's instructions. Transfections were performed 48 hours after plating cells in 24 or 6 well plates and aromatase activity was assayed 48 hours post-transfection. Transfections were optimized for cell plating density, vector DNA added and transfection reagent volume. Optimal conditions (per 2 cm^2 well) were 2.5 ul of lipofectamine, 1 μg pCI-neo vector and 1 ng pRL-TK vector transfected into 2×10^5 HEK 293 cells. For the

kinetic experiments, cells were plated into 6 well plates. For all other experiments, cells were plated into 24 well plates.

Transfected cells were incubated with serum free media with 1 mg ml^{-1} bovine serum albumin (Sigma, St. Louis MO) and either [1β-^{3}H]-androstenedione or [1β,2β-^{3}H]-testosterone (NEN Life Sciences, Boston MA) in a 37°C and 5% $C0_2$ incubator. The substrates were diluted with cold substrate to a specific activity of 8 or 5.18 uCi $nmol^{-1}$ in ethanol for testosterone or androstendione, respectively. The volume of ethanol added to each well was 2% of the total media volume. Additional triplicate wells were incubated with tritiated water to correct for the extraction efficiency and possible water loss during incubation. After incubations were complete, the media was collected for determination of aromatase activity. One volume of media was added to a half volume of ice cold 30%TCA and the remaining media was flash frozen in liquid nitrogen. The cells were washed with PBS and harvested preparation of cell lysates and measurement of luciferase activity using either the dual or Renilla luciferase assay kit (Promega, Madison WI). Cell lysates were prepared according to manufacturer's instructions. Luciferase activity was measured in 20 ul of cell lysate on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) after a 2 s delay and a 10 s integration time.

Aromatase activity was determined using the tritiated water release assay (Lephart and Simpson 1991). Briefly, media/TCA aliquots were centrifuged to precipitate any protein and one volume was extracted with 2.5 volumes of chloroform. The aqueous phase was mixed with an equal volume of 5% charcoal, 0.5% dextran solution and centrifuged. An aliquot of the aqueous phase was mixed with scintillation fluor (Scintiverse BD, Fisher Scientific, Hampton, NH) and counted on a scintillation counter. Aromatase activity was calculated by converting dpms counted into pmoles water produced per well after correcting for label (testosterone), extraction efficiency and decay. Aromatase activity was normalized with Renilla luciferase activity in each well. Each dose or time point was done in triplicate wells and averaged.

To determine the kinetics of aromatase activity, cells were plated and transfected in 6 well plates. Transfected cells were exposed to 150 nM substrate and media was sampled from each well at 30 minutes, 1,2, 3, 4, and 6 hours. 50% of media was removed

from each well by the 6 hour time point. For substrate saturation experiments, transfected cells were exposed to 0 to 150 nM substrate for 2 hours. K_m and V_{max} were determined with a lineweaver-burke plot, where the V_{max}= (y intercept)$^{-1}$ and K_m= slope $\times$ V_{max}. For the inhibition experiments, transfected cells were exposed to 63.5 nM androstenedione and 0 to 100 nM letrazole (Novartis Pharmaceuticals Corp, East Hanover NJ) for 2 hours.

Results

Dolphin Aromatase Sequence

A white-sided dolphin (*Lagenorhynchus acutus*) *CYP19* sequence (GenBank accession number (AY341076) representing approximately 2000 base pairs with an open reading frame of 1509 base pairs was obtained from ovarian tissue. The sequence was confirmed with • 6-fold bi-directional coverage within the coding sequence. The full-length cDNA has been cloned and sequenced. The dolphin *CYP19* was most similar to the cow *CYP19* (Table 3). Among several mammalian full length coding sequences, dolphin CYP19 ranged from 81.1 - 93.9% and 78.0 - 92.3% identity, based on nucleotide and amino acid sequence, respectively (Table 3).

Phylogenetic Analysis

After exclusion of poorly aligned regions, the final aromatase amino acid alignment included 436 characters, of which 282 were parsimony informative and 115 constant. The length of the MC^3 burn-in was 8,000 of 10,000,000 generations, resulting in a sample of 99,920 trees for estimation of posterior probabilities. Replicate analyses provided very similar estimates of posterior probabilities. Overall resolution of CYP19 phylogeny was high, as indicated by posterior probabilities (Fig. 1). The dolphin sequence grouped with aromatases from the artiodactyls (Figure 1) as expected based on phylogenetic relationships within the Mammalia.

DIVERGE Analysis

Functional divergence over the taxonomic groups represented in Figure 1was examined in context of the tree topology, using the DIVERGE software and the rabbit 2C5 crystal structure, the closest cytochrome P450 crystal structure available for modeling aromatase (Williams *et al.* 2000). For this analysis, at least 4 proteins are

required for each grouping, thus the functional divergence of the pig sequences could not be specifically tested. Instead, large taxonomic groups were designated: mammals, turtle/bird/reptile, amphibians, and fish (either as one group, or divided into fish a and b forms). In nearly all cases, there was no functional divergence detected between these groupings (Table 4). The only significant divergence from $\theta = 0$ found was between fish and mammals, although this was marginal (i.e. $\theta < 0.50$, Table 4). This difference was found whether the fish were grouped as a whole or divided into a and b forms. When divergent residues were plotted across the alignment, they appeared randomly distributed across the entire protein (data not shown). The divergent residues did not cluster relative to the presumed secondary structure of the protein.

Transient Transfections

The dolphin *CYP19* transfection protocol was demonstrated to yield a functional CYP19 by the transfectant-dependant appearance of estradiol, detected by radioimmunoassay, in the media of transfected cells incubated with unlabelled testosterone (data not shown). In the optimized transfection system, the rates of substrate conversion were linear up to 4 hrs or 3 hrs for testosterone or androstenedione, respectively (Figure 2). A higher rate of substrate conversion was seen with androstenedione at all time points. Substrate saturation experiments confirmed that the K_m was higher for testosterone than androstenedione. The dolphin CYP19 K_m and V_{max} were 75nM and 19.4 pmoles•2 hr^{-1} for testosterone and 63.5 nM and 25.9 pmoles•2 hr^{-1} for androstenedione (Figure 3 and Table 5).

Letrazole is a specific inhibitor of aromatase. The addition of 100 nM letrazole inhibited 96% of the dolphin aromatase activity in the presence of 63.5 nM androstenedione (data not shown). The presence of estradiol in the media of initial transfection experiments and the ability of letrazole to completely block aromatase activity in this system, confirms that tritiated water production was specifically caused by aromatase activity.

Discussion

Phylogenetic Analysis

The aromatase gene is well conserved across mammalian species, with nucleotide identity greater than 80% between dolphin and other mammals (Table 3) as is expected based on previous studies (Tchoudakova and Callard 1998; Conley *et al.* 2001a). Based on both molecular phylogeny and fossil evidence, cetaceans are thought to be most closely related to the ruminant artiodactyls (Gatesy *et al.* 2001). In agreement with species phylogeny, the dolphin aromatase was most closely related to the bovine, sheep and goat aromatases (Figure 1). The porcine aromatases were in a sister grouping to the cetartiodactyl clade, which also reflects current molecular evidence of the evolution of the ungulates (Gatesy *et al.* 2001). Interestingly, all porcine aromatase sequences were more closely related to each other than to any other aromatase with a very high posterior probability, suggesting that gene duplication events occurred only in this lineage, as has been previously suggested (Graddy *et al.* 2000; Conley *et al.* 2001a). Further taxon sampling within the ungulates will be able to specifically test this hypothesis. Overall, these results were in agreement with phylogenetic reconstructions based on a maximum likelihood analysis of nucleotide sequences from a more limited mammalian alignment (data not shown).

The aromatase phylogenetic tree follows current knowledge of species phylogeny. Within the mammals, clades with lower posterior probabilities contain species that are traditionally difficult to place in mammalian evolution, including the rabbits being potential sister taxa to the rodents (glires), which is supported by morphological evidence but is not always supported by molecular phylogenetics (de Jong 1999). Perrisodactyls (which include horse), are also difficult to place in the mammalian tree and are not always sister taxa to the cetartiodactyls (Shoshani and McKenna 1998; Liu and Miyamoto 1999). Areas of the aromatase tree that have good support are those clades that are well resolved in other studies.

Areas of well understood vertebrate phylogeny had very high support in the aromatase tree (as indicated by posterior probabilities of 1.00), including the grouping of all fish, amphibian, turtle/reptile/bird and mammal into appropriate clades. Posterior

probabilities are generally higher than non-parametric bootstrap estimates (Huelsenbeck *et al.* 2002). However, posterior probabilities of 1.00, as seen on several nodes of the aromatase tree, should represent nodes that would also have strong support under bootstrap analysis. Amongst the fish, the ovarian (CYP19a) and brain (CYP19b) aromatase proteins formed separate clades, indicating a duplication event early in fish diversification, at least prior to the bony fish. These data suggest that nuclear genes that encode for proteins which carry out highly conserved functions, such as aromatase, may be useful for phylogenetic reconstructions among otherwise difficult to resolve groups. The evolution of mammals, which is referred to as an "unresolved bush", has been difficult to reconstruct and there is little agreement on many interordinal relationships studied with typical ribosomal and nuclear genes. Proteins such as aromatase may be helpful in resolving such discrepancies, although much more taxon sampling would be required to test if the phylogenetic signal is useful for resolution of this part of evolutionary history.

Transient Transfections

The dolphin aromatase was transiently transfected into HEK 293 cells. This cell line does not metabolize androstenedione nor testosterone to any detectable degree over 6 hours or more of culture (Corbin *et al.* 1999a), making it a particularly useful line for aromatase transfection studies. HEK 193 cells have been used for both transient and stable transfections of porcine aromatase (Corbin *et al.* 1999a; Corbin *et al.* 2001).

Substrate conversion was linear for at least 2 hours with both androstenedione and testosterone. This is similar to the time course seen with porcine aromatase expressed in the same cell system (Corbin *et al.* 1999a). Additional NADPH-cytochrome P450 reductase was not used in these experiments. Work with pig aromatases transfected into HEK 293 cells showed that additional reductase increased the aromatase activity (Corbin *et al.* 2001), indicating that reductase may have been limiting in these experiments. However, the K_m of cetacean aromatase was very similar to that reported for other mammalian aromatases (see Table 5, Ackerman *et al.* 1981; Corbin *et al.* 1988; Pompon *et al.* 1989; Zhou *et al.* 1990; Corbin *et al.* 1999a; Stresser *et al.* 2000). Thus, the functional characteristics of cetacean aromatase seem very similar to all other mammalian

aromatase proteins studied to date, except the pig placental aromatase. Pig varies from other mammals in that they have three distinct aromatase genes (Choi *et al.* 1996; Corbin *et al.* 1999b; Graddy *et al.* 2000) instead of one gene with tissue specific promoters (Conley *et al.* 2001a; Simpson *et al.* 2002). The pig placental aromatase has different functional characteristics from other mammalian aromatases, as seen by slower metabolism of testosterone (i.e. lower V_{max}), lower K_m values for androgens in general, and a higher affinity for testosterone than for androstendione in the placenta, compared to the gonadal form (Corbin *et al.* 1999b; Corbin *et al.* 2001). Pig gonadal, but not placental, aromatase is sensitive to the typical aromatase inhibitor etomidate (Corbin *et al.* 1999b) and the placental form has a higher dependence on reductase activity (Corbin *et al.* 2001). Thus, it appears that the pig ovarian form, but not the placental, is like other mammalian aromatases, including the white-sided dolphin.

DIVERGE Analysis

Diversification of drug metabolizing CYP enzymes has been suggested to be determined predominantly by six substrate recognition sites (SRSs) and it has been shown that the SRSs are more variable than the rest of the sequence in the CYP2 family (Gotoh 1992). However, in aromatase, SRS 5 and 6 cover regions with complete identity between pig placental and gonadal forms (Conley *et al.* 2001b) suggesting that these SRSs may be invariant in aromatase. Regions of high similarity in aromatases from diverse taxa including mammals, birds and both fish forms, occur in the heme binding region, the I helix region (which includes SRS4), and in an aromatase specific conserved sequence (Graham-Lorence *et al.* 1995; Tchoudakova *et al.* 1998).

In a recent CYP3 family analysis, the boundaries of the several SRSs were found to be too small to account for functional differences among the proteins while other SRSs were not functionally divergent (McArthur *et al.* 2003). However, with aromatase, there is little functional divergence over all vertebrates. There was no significant functional divergence in aromatase between either mammals or fish and the amphibians or turtle/reptile/bird group, as detected by DIVERGE. The fish a and b forms were not significantly different from each other. θ was significantly greater than zero only in the comparison between the mammal and fish aromatases, no matter whether the comparison

included all fish, CYP19a, or CYP19b. The functional differences between mammals and fish aromatases were marginal ($\theta < 0.5$, Table 4) and the distribution of functionally diverging residues was random across the protein (data not shown). This is in strong contrast to the CYP3 family where the functional differences between taxa were very strong and large clustering of divergent residues enabled the identification of regions of the protein that were distinct (McArthur *et al.* 2003). Since DIVERGE requires at least four sequences per grouping, it was impossible to test the functional divergence within the pig aromatases specifically. It would be very interesting to repeat this analysis when sufficient aromatase sequence information was available from other pig species.

The CYP3 family represents a family of genes with extraordinary functional diversity. In contrast, the CYP19 family has only 5 genes (ovarian form, embryo and placental forms from pig and two fish forms), all with the same few substrates. As such, it is not surprising that these analyses did not see a large degree of functional diversity like that in the CYP3 family. The lack of clustering of divergent residues demonstrates how strongly aromatase is conserved over the entire protein. Different aromatases appear to differ at the transcriptional level only and not at the level of protein function. Selection for conservation of function is strong throughout this protein and the observed sequence divergence is functionally neutral.

In support of this, the observed differences in substrate specificity appear minimal, as seen in very similar substrate K_m among mammalian aromatases (Table 5). One might expect that the catalytic properties and substrate preference would be similar not only among mammals, but also among birds, reptiles, turtles and amphibians. Fish aromatases have a preference for testosterone over androstenedione (Zhao *et al.* 2001), in contrast to all mammalian aromatases studied to date except the placental form in pig (Corbin *et al.* 1999a). This subtlety is most likely below the detection limit of DIVERGE. As such, it is probable that only site-directed mutagenesis studies will be able to determine residues responsible for this bias.

The phylogenetic and functional divergence analyses, along with existing information on the few aromatases studied catalytically to date, indicate that aromatase is an extraordinarily well conserved protein within the vertebrates. With ongoing

identification of aromatase activity and *in vivo* estrogen production in invertebrates (Callard *et al.* 1984; Matsumoto *et al.* 1997; Le Curieux-Belfond *et al.* 2001; Oberdorster and McClellan-Green 2002; Santos *et al.* 2002; Twan *et al.* 2003), it is possible that this protein, and its function in the production of estrogens, has been conserved outside of the vertebrates.

That aromatase is so important in mammalian physiology is demonstrated by the dearth of human subjects with mutations in the *CYP19* gene. Studies in traditional model organisms should be very applicable for extrapolation to other species. Ongoing efforts to study aromatase in novel species should focus on aromatase copy number, the promoter regions, and expression profiles, to better understand the sites of aromatase expression and similarity in transcriptional control across species. For wildlife species, such as marine mammals where experimental work is intractable or unethical, aromatase studies in model organisms should help elucidate the control, function and importance of this protein in both normal physiology and pathophysiological conditions. Our work shows this is specifically true for white-sided dolphins.

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Table 1. Primer Sequences and Fragment Sizes for RT-PCR, RACE and Amplification of the Full-length Sequence of White-sided Dolphin Aromatase.

Primer Name	Primer Sequence	Fragment Size
Internal set 1		
CYP19_672-691F	5'-GCAAGCTCTCCTYMTCAAAC-3'	~700 bp
CYP19_1353-1373R	5'-AAKCGTCTCARAAGTGTRACC-3'	
Internal set 2		
CYP19_2-23F	5'-TGNTTTTGGAAATGCTGAACCC-3'	~830 bp
CYP19_810-839R	5'-TCCATRBDGTCTTCCAGTTTC-3'	
3' RACE primers		
CYP19_3'RACE1	5'-ACAAGCTCTTCCTGGGGAGCCCCTTGG-3'	~1.5 kb, ~3 kb
CYP19_3'RACE2	5'-GGATGATGTCATCGATGGCTACCCAGTG-3'	~900 bp, ~1.2 kb
5' RACE primers		
CYP19_5'RACE	5'-CAACAGGCGCAACTTCGGTCACCATGC-3'	~700 bp[1]
CYP19_5'RACE2	5'-GTCCAAGGGGATCCCCAGGAAGAGCTTG-3'	None
CYP19_5'RACE3	5'-CCTTTCTCATGCATGCCGATGCACTGC-3'	None
Full length primers		
Fullcds_CYP19F	5'-CGCAAGATGGTTTTGGAAGTGC-3'	1544 bp
Fullcds_CYP19R	5'-AGGGCAGGGACTGACCAAAC-3'	

[1]fragments were generated with a nested PCR, using the RACE reaction generated with primer CYP19_5'RACE2 and CYP19_5'RACE3.

Table 2. GenBank accession numbers of cytochrome P450 (CYP) sequences used in the present study.

Species	Accession Number
Alligator CYP19 (*Alligator mississippiensis*)	AAK31803
Atlantic Halibut CYP19A (*Hippoglossus hippoglossus*)	CAC36394
Bastard Halibut CYP19A (*Paralichthys olivaceus*)	BAA74777
Catfish CYP19A (*Ictalurus punctatus*)	AAB32613
Catfish CYP19B (*Ictalurus punctatus*)	AAL14612
Chicken CYP19 (*Gallus gallus*)	A31916
Cow CYP19 (*Bos Taurus*)	P46194, Z32741[1]
Dolphin CYP19 (*Lagenorhynchus acutus*)	AY341076
European Seabass CYP19A (*Dicentrarchus labrax*)	CAC21712
Fathead Minnow CYP19B (*Pimephales promelas*)	CAC38767
Frog CYP19 (*Xenopus laevis*)	BAA90529
Gilthead Sea Bream CYP19A (*Sparus aurata*)	AAL27699
Goat CYP19 (*Capra hircus*)	AAN23836
Goldfish CYP19A (*Carassius auratus*)	AAC14013
Goldfish CYP19B (*Carassius auratus*)	BAA23757
Horse CYP19 (*Equus caballus*)	AAC04698, AF031520[1]
Human CYP19 (*Homo sapiens*)	CAA68807, Y07508[1]
Japanese Quail CYP19 (*Coturnix japonica*)	AAN04475
Macaque CYP19 (*Macaca fascicularis*)	BAB64454
Marmoset CYP19 (*Callithrix jacchus*)	AAK58465
Medaka CYP19A (*Oryzias latipes*)	BAA11657
Mouse CYP19 (*Mus musculus*)	NP_031836, G3046857[1]
Mozambique Tilapia CYP19A (*Oreochromis mossambicus*)	AAD31031
Mozambique Tilapia CYP19B (*Oreochromis mossambicus*)	AAD31030
Newt CYP19 (*Pleurodeles waltl*)	AAM95462
Nile Tilapia CYP19A (*Oreochromis niloticus*)	P70091
Nile Tilapia CYP19B (*Oreochromis niloticus*)	AAG49480
Pig CYP19 Type I (*Sus scrofa*)	AAB51388, U92246[1]
Pig CYP19 Type II (*Sus scrofa*)	AAB61697, U52142[1]
Pig CYP19 Type III (*Sus scrofa*)	2210279A
Rabbit CYP19 (*Orycholagus cuniculus*)	CAA92574, Z70301[1]
Rainbow Trout CYP19A (*Oncorhynchus mykiss*)	1806325A
Rainbow Trout CYP19B (*Oncorhynchus mykiss*)	CAC84574
Rat CYP19 (*Rattus norvegicus*)	A36121, M33986[1]
Red Sea Bream CYP19A (*Pagrus major*)	BAB82524
Sheep CYP19 (*Ovis aries*)	CAB40563
Stingray CYP19 (*Dasyatis sabina*)	AAF04617
Turtle CYP19 (*Tarchemys scripta*)	AAG09376
Zebrafinch CYP19 (*Taeniopygia guttata*)	AAB32404
Zebrafish CYP19A (*Danio rerio*)	AAK00643
Zebrafish CYP19B (*Danio rerio*)	AAK00642

[1]nucleotide sequences used for primer design and nucleotide phylogeny (data not shown).

Table 3. Percent Sequence Identity of Aromatase between White-sided Dolphin and Selected Other Mammalian Species.

Sequence	% Identity				
	Rat	Rabbit	Horse	Human	Cow
Amino Acid	78.0	84.5	78.7	84.7	92.3
Nucleotide	81.1	85.5	85.7	87.7	93.9

Table 4. Tests of the null hypothesis of no change in site-specific evolutionary rates among the four largest CYP19 sub-clades (containing more than four proteins, see Fig. 1), as implemented by the computer program DIVERGE. Measures of the coefficient of evolutionary functional divergence (θ) and their standard error are presented above the diagonal. Increasing values of θ reflect increasing functional divergence. Likelihood ratio test observed values for the test of the null hypothesis of θ = 0 are presented below the diagonal. Scores with significant rejection ($P < 0.05$) of the null hypothesis are marked with an asterisk.

	Mammal CYP19	**Turtle, Reptile, Bird CYP19**	**Fish CYP19A**	**Fish CYP19B**
Mammal CYP19	-	0.12 ± 0.11	0.48 ± 0.07	0.41 ± 0.07
Turtle, Reptile, Bird CYP19	1.25	-	0.20 ± 0.13	0.13 ± 0.16
Fish CYP19A	45.45 *	2.47	-	0.00 ± 0.02
Fish CYP19B	30.86 *	0.73	0.00	-

Table 5. K_m Values for Aromatase in a Variety of Mammalian Species.

Species, Tissue	K_m (nM)	Substrate	System
White-sided dolphin, ovary	75	Testosterone	Transient transfection
	63.5	Androstenedione	
Human, adipose [1]	25	Androstenedione	Stromal cells in culture
Human, placental	55[2]	Testosterone	Stable transfection
	43[3]	Testosterone	Baculovirus transfection
	50[2]	Androstenedione	Stable transfection
	39.9 – 57.8[4]	Androstenedione	Stable transfection, multiple cell lines
	34[5]	Androstenedione	Yeast expression
Pig, placental[6]	33	Testosterone	Stable transfection
	77	Androstenedione	
Pig, ovary [6]	116	Testosterone	Stable transfection
	104	Androstenedione	

[1] (Ackerman *et al.* 1981), [2] (Corbin *et al.* 1988), [3] (Stresser *et al.* 2000), [4] (Zhou *et al.* 1990), [5] (Pompon *et al.* 1989), [6](Corbin *et al.* 1999a).

Figure 1. Phylogeny of Vertebrate Aromatase. The phylogenetic tree with the highest posterior probability score found by MC^3. A better tree may exist as MC^3 is a stochastic sampling method. Bayesian posterior probabilities for each node are superimposed upon the tree. The tree is rooted using the stingray CYP19 sequence. Horizontal branch lengths are representative of evolutionary change.

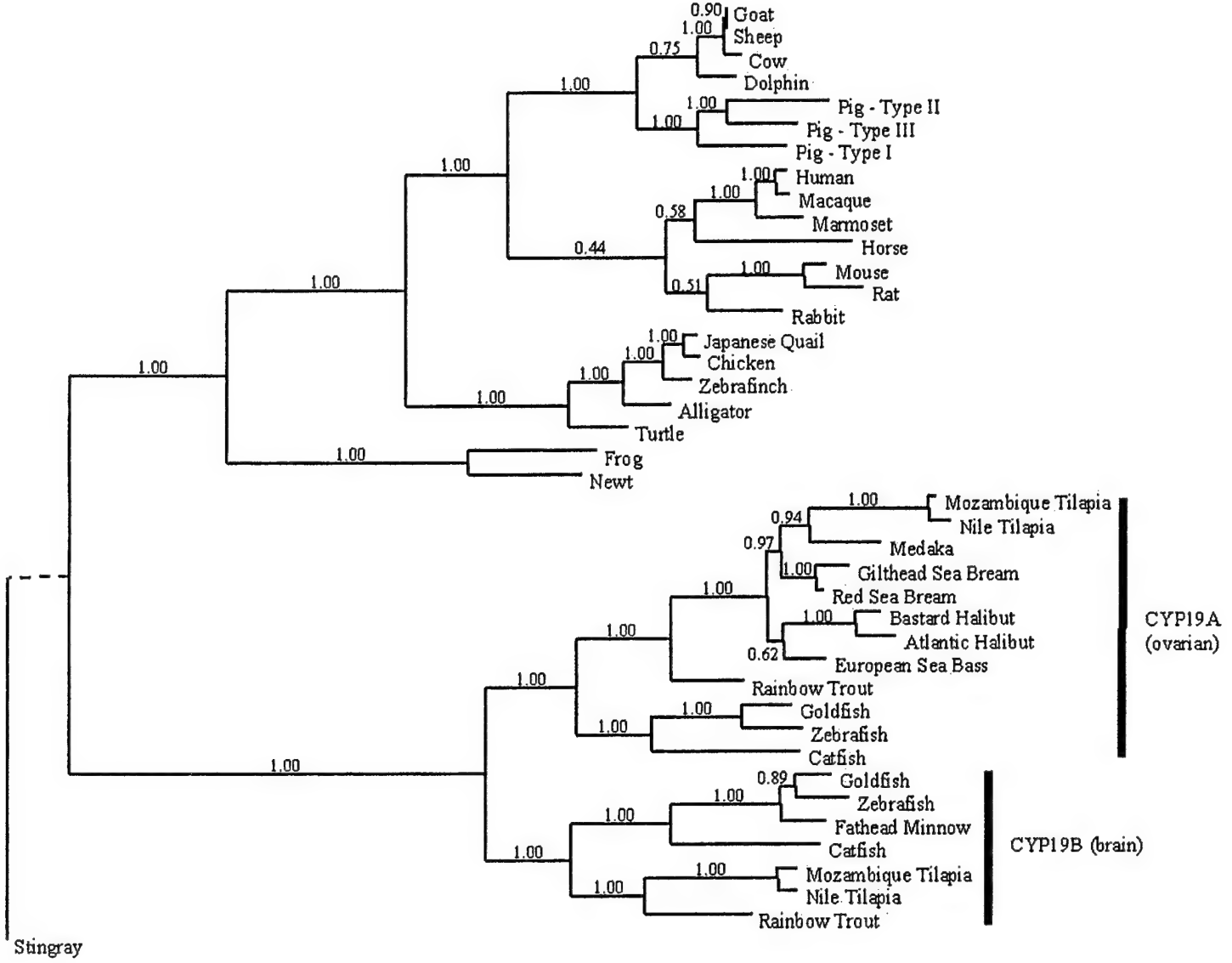

Goat
Sheep
Cow
Dolphin
Pig - Type II
Pig - Type III
Pig - Type I
Human
Macaque
Marmoset
Horse
Mouse
Rat
Rabbit
Japanese Quail
Chicken
Zebrafinch
Alligator
Turtle
Frog
Newt
Mozambique Tilapia
Nile Tilapia
Medaka
Gilthead Sea Bream
Red Sea Bream
Bastard Halibut
Atlantic Halibut
European Sea Bass
Rainbow Trout
Goldfish
Zebrafish
Catfish
Goldfish
Zebrafish
Fathead Minnow
Catfish
Mozambique Tilapia
Nile Tilapia
Rainbow Trout
Stingray
CYP19A (ovarian)
CYP19B (brain)

Figure 2. Tritiated Water Release From Testosterone and Androstenedione Over Time in Cells Transiently Transfected with Dolphin Aromatase. Assays were performed in triplicate as described in materials and methods.

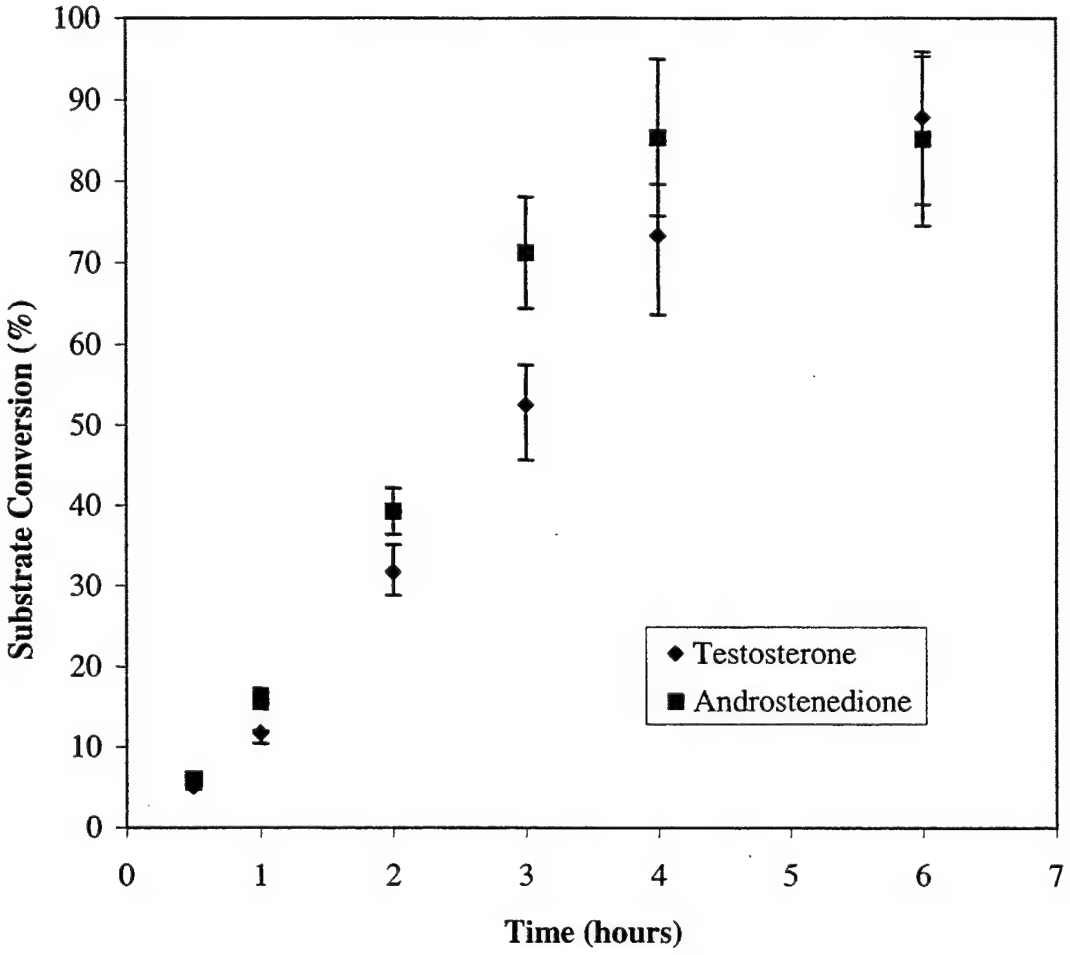
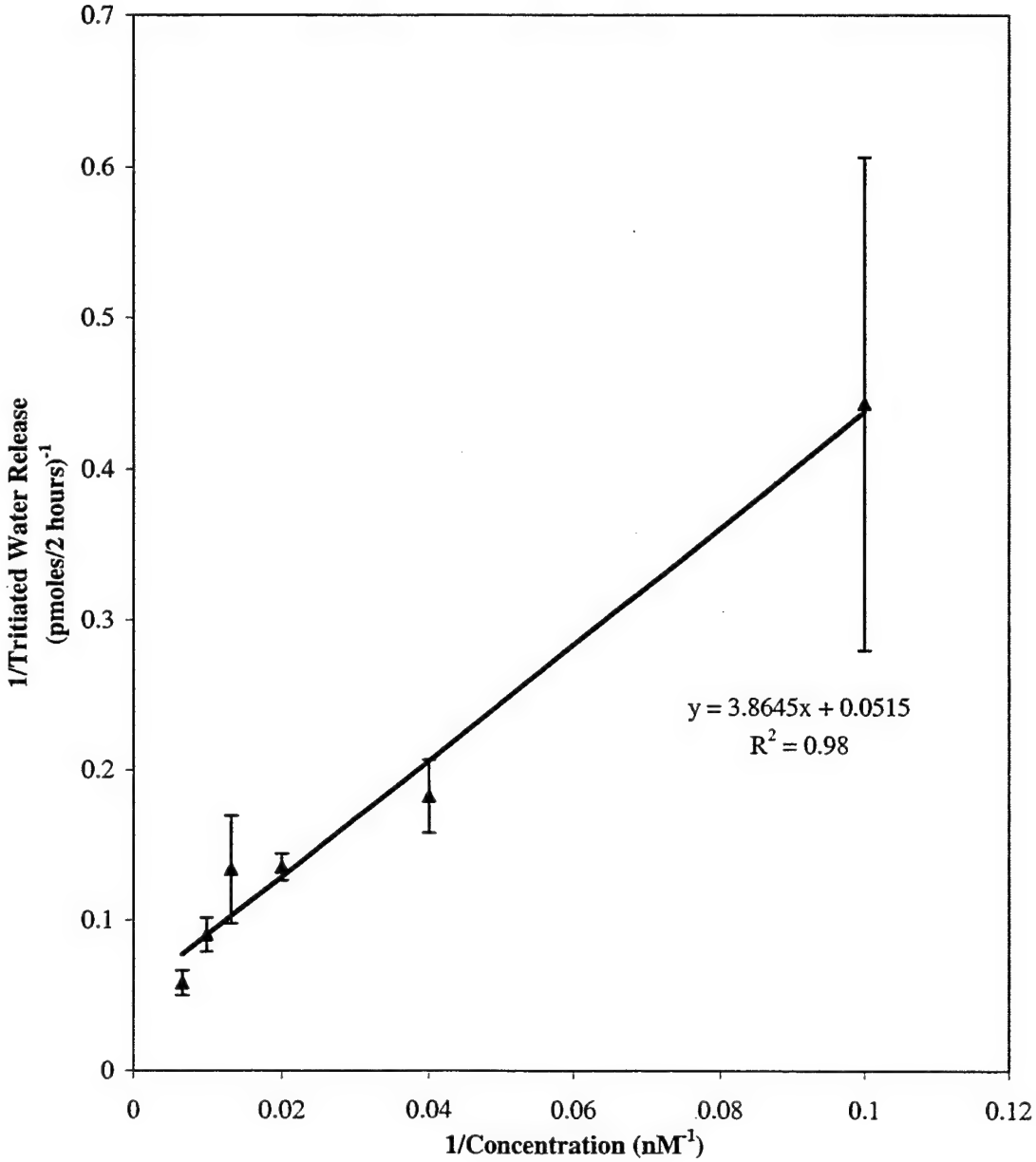
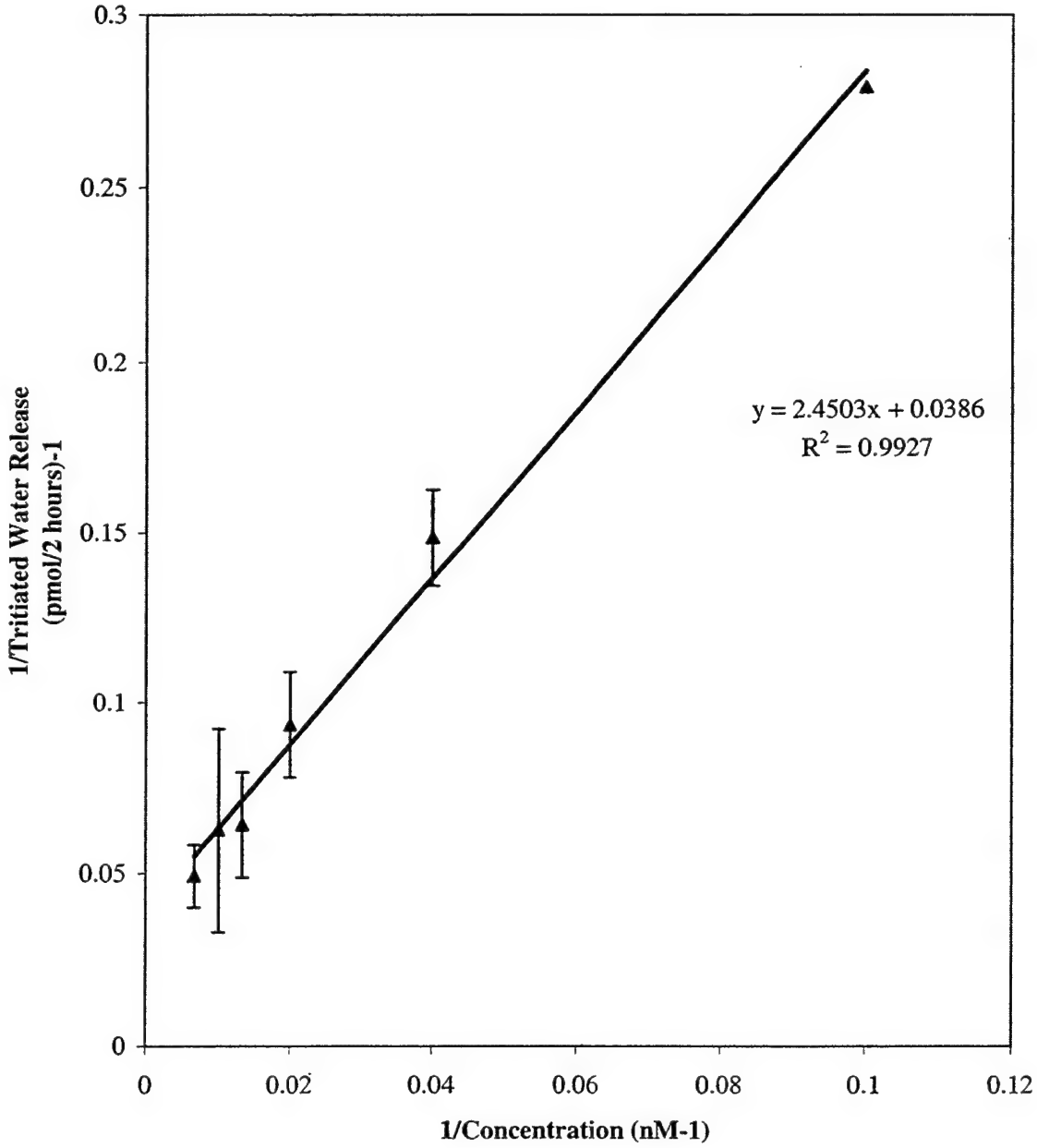

100
90
80
70
60
50
40
30
20
10
0
Substrate Conversion (%)
0
1
2
3
4
5
6
7
Time (hours)
Testosterone
Androstenedione

Figure 3. Aromatase Activity at Varying Concentrations of Testosterone (A) and Androstenedione (B) in Cells Transiently Transfected with Dolphin Aromatase. Assays were performed in triplicate as described in materials and methods. Aromatase activity was measured with the tritiated water release assay.

A


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.02
0.04
0.06
0.08
0.1
0.12
1/Tritiated Water Release (pmoles/2 hours)-1
1/Concentration (nM-1)
y = 3.8645x + 0.0515
R2 = 0.98

B


0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.02
0.04
0.06
0.08
0.1
0.12
1/Tritiated Water Release (pmol/2 hours)-1
1/Concentration (nM-1)
y = 2.4503x + 0.0386
R2 = 0.9927

Chapter 6. Summary and Conclusions

Ongoing efforts to determine contaminant concentrations in cetaceans have given us a wealth of information on the exposure of cetacean populations to PCBs, DDT and other chlorinated pesticides. These studies show that odontocetes, which feed higher in the food chain, have higher contaminant concentrations than mysticetes (O'Shea and Brownell 1994). Coastal populations, which are closer to the sources of anthropogenic contaminants, are thought to be at greater risk than offshore populations. We understand the impacts of age and sex upon contaminant burdens. For male cetaceans, contaminants usually accumulate over time. Females have depuration associated with reproduction and lactation and contaminant concentrations decline throughout the reproductive years (Aguilar and Borrell 1988). Studies have identified three cetacean populations that have comparatively high contaminant concentrations; the St. Lawrence beluga whale (Muir *et al.* 1996), the Mediterranean striped dolphin (Aguilar and Borrell 1994; Guitart *et al.* 1996), and the northwest Pacific killer whale (Ross *et al.* 2000). The St. Lawrence beluga suffers from an unusually high prevalence of tumors and lesions (Martineau *et al.* 1994) and the Mediterranean striped dolphin has been subject to a large mass mortality associated with a viral infection (Domingo *et al.* 1990). In contrast, there are currently no data to suggest that the northwest Pacific killer whale suffers from unusual or obvious toxic impacts, even though they have higher concentrations than the St. Lawrence beluga. Contaminant analysis alone cannot indicate which species are most at risk of toxicity because species vary in their response to contaminants. To assess the sensitivity or responsiveness of a species to contaminants, we must measure biological responses to contaminants in cetaceans and relate this response to contaminant concentrations. Comparing the response to contaminant loads in multiple species will allow the determination of relative sensitivities of cetaceans to certain contaminants.

CYP1A1 Expression in Cetaceans

CYP1A1 Induction is Conserved across Taxa

The induction of CYP1A1 after exposure to certain PHAH and PAH contaminants is a well understood and characterized response in mammalian species (Whitlock 1999). This response pathway offers a good system to understand the inherent differences in

species responsiveness to PHAH and PAH exposures. Cytochrome P450 1A enzymes are present in vertebrates, and certain aspects of these enzymes are conserved. Although some of the details, such as the number of AHRs present in a species (Hahn 2002), may vary, the induction of CYP1A1 by PAHs and PHAHs through the AHR is well understood (Whitlock 1999). CYP1A1 is induced by and metabolizes similar suites of compounds in all vertebrate taxa. Locations and relative intensities of CYP1A1 expression are conserved in mammalian, and indeed sometimes in all vertebrate taxa. Vertebrates generally show high levels of CYP1A1 induction in the liver. In mammals, hepatic CYP1A1 has a zonal expression pattern in which panlobular expression of CYP1A1 typically is seen only in highly induced organisms (Oinonen and Lindros 1998). This conservation of function, induction pathway, and in some cases expression patterns, allows us to infer the biochemical response of species where experimental exposures are lacking. Since the induction of CYP1A1 has been related to other toxic effects in rodents (Safe 1987; Safe 1990), determining the relative degree of CYP1A1 induction may also indicate the relative sensitivity to PHAH and PAH toxicity. As a first step towards understanding which cetaceans are most at risk for PHAH induced toxicity, the goals of this thesis were to examine (i) the major sites of expression of CYP1A1 in different cetacean species, (ii) the relationship between PHAH contaminants and CYP1A1 expression, and (iii) the importance of life-history parameters such as age and sex on CYP1A1 expression in cetaceans.

Cross-species Comparisons and Apparent Differences in Sensitivity

Cross-species comparisons may be useful to highlight the sensitivity of cetacean species for induction of CYP1A1. Expression patterns of CYP1A1 in internal organs were compared to conserved patterns of expression in mammals. It was inferred that Arctic beluga were highly induced despite having some of the lowest contaminant exposures among the odontocetes (Chapter 2). Widespread, high level expression of CYP1A1 has previously been seen only in highly induced animals. Yet, beluga from both the St. Lawrence Estuary and the Arctic had a similar widespread, high level expression pattern in multiple internal organs. High level, panlobular expression in liver and the expression of CYP1A1 in endothelial cells throughout most organs suggest that even at

the low doses in the Arctic, beluga whales are strongly induced. The implication that beluga are very sensitive to PHAH contaminants supports the link between contaminants and the pathologies seen in the St. Lawrence beluga whale.

Measures of CYP1A1 in white-sided dolphin (Chapter 3) were compared to other species, particularly the beluga whale. As has been shown for beluga whale (White *et al.* 1994), hepatic CYP1A1 induction, in this case measured as catalytic function (EROD activity), was highly correlated to the concentration of Σmono-*ortho* PCBs in blubber. Yet hepatic CYP1A1 levels and EROD activity were markedly lower in white-sided dolphin than in beluga whale in spite of the fact that beluga whale are exposed to lower PCB concentrations. This difference most likely indicates a difference in the sensitivity to induction of these species to PHAHs, and that white-sided dolphin may require much higher exposures to be induced to the same extent as a beluga whale. Similarly, comparisons of white-sided dolphin with pilot whale and harbour porpoise from the same region indicate that pilot whale may be the most sensitive of these three species. These types of comparisons should allow us to identify those cetacean species that are most sensitive to PHAH contaminants and, with contaminant concentrations, infer which populations are at the highest risk of PHAH induced toxicity.

PAH Exposure is Unknown

A caveat for most of this research is that PAH measurements are lacking for almost all cetacean populations, although PAHs are detectable in blubber of cetacean species (Marsili *et al.* 2001). Many PAHs are rapidly metabolized which makes determining all but the most recent exposure difficult. CYP1A1 is induced by both PHAHs and PAHs, yet only PCBs are typically measured in cetacean tissues. Other measures of PAH exposure, such as DNA adduct concentrations, have been determined in beluga (Ray *et al.* 1991; Mathieu *et al.* 1997), but this measure is dependant not only on the exposure, but also the capacity to metabolize PAHs, and DNA repair mechanisms. Although DNA adducts are used as a proxy measure for exposure, they could not be easily used as a marker of exposure when comparing across species, particularly in cetacean species where rates of PAH metabolism and DNA repair rates are unknown. Estimates of PAH exposure may be possible by determining PAH concentrations in prey

items, although changes in composition of diet over time would make it difficult to determine exposures to individual animals. Small scale regional differences in PAH concentrations are possible, making assumptions of PAH exposures difficult even on a regional scale. Without an adequate understanding of PAH burdens, it is difficult to be certain if a species is more responsive to contaminants. Differences in CYP1A1 expression could simply reflect higher, unmeasured PAH exposures.

The Impact of Life-history Parameters

The impacts of age and sex on CYP1A1 expression appear to be minimal. CYP1A1 expression does not appear to differ between male and female bottlenose dolphin and males and females fall on the same exposure-response curve in beluga whale (White *et al.* 1994), white-sided dolphin (Chapter 3), and bottlenose dolphin (Chapter 4). Age or length, which can be used as a proxy for age, is not well correlated with CYP1A1 expression in either white-sided dolphin (Chapter 3) or bottlenose dolphin (Chapter 4). Contrary to all other species examined to date, contaminant concentrations appear to decrease with age in male white-sided dolphin, even though a strong positive correlation between PCB concentrations and CYP1A1 expression was found (Chapter 3). This is strong evidence that although age may be related to contaminant accumulation, it is not an important variable for CYP1A1 expression. Contaminant concentration, and not life-history parameters, is the important determinant of CYP1A1 induction.

CYP1A1 Expression and Mono-ortho PCB concentrations are Correlated

A strong correlation between hepatic CYP1A1 expression and mono-*ortho* PCB concentrations has been established for beluga whale, although these animals were underweight for their length, suggestive of significant lipid mobilization (White *et al.* 1994). Hepatic EROD activity, which is catalyzed by CYP1A1, was strongly correlated to mono-*ortho* PCB concentrations in the blubber of white-sided dolphins (Chapter 3), a relationship between liver CYP1A1 and mono-*ortho* PCBs like that seen in beluga whale. A weaker correlation was seen between mono-*ortho* PCB concentrations and endothelial CYP1A1 expression in the dermis (or blubber) of bottlenose dolphin (Chapter 4). Although this correlation depended strongly upon just two animals with higher contaminant concentrations, it confirms a relationship between CYP1A1 expression and

contaminants in cetacean integument (Fossi *et al.* 1992; Marsili *et al.* 1998) and supports the usefulness of biopsies for examining CYP1A1 induction in free-ranging animals. Ongoing efforts should focus on replicating this relationship in other species and obtaining more biopsies of older male bottlenose dolphin, whose higher contaminant concentrations would allow us to fill in the PCB – CYP1A1 response curve.

Future Work with CYP1A1 in Cetaceans

The assessment of PAH exposures in cetaceans is needed so that we can understand the exposure of cetaceans to the full range of inducing compounds. PAHs can be measured in cetacean blubber (Marsili *et al.* 2001) and presumably in other tissues, yet this is rarely done in contaminant studies. That PAHs are more readily metabolized than PHAHs means that only the current exposure can be assessed and that blubber PAHs likely represent the non-metabolized congeners only. Interpretation of CYP1A1 levels in cetaceans, particularly when comparisons are made between species with known PCB exposures, require adequate measures of PAHs to ensure that differences in CYP1A1 expression are not simply a reflection of differences in PAH exposures. CYP1A1 levels in integument biopsies from the north Atlantic right whale are not well correlated with PCB concentrations, but do appear related to PAH concentrations found in copepods, their main prey (M. Moore, pers. com.). This study demonstrates the possible importance of PAH exposures for CYP1A1 induction in cetaceans.

Although CYP1A1 induction has been correlated with total or Σmono-*ortho* PCBs, not all mono-*ortho* PCBs are positively correlated with CYP1A1. A subset of mono- and non- *ortho* PCB congeners may be primarily responsible for CYP1A1 induction. Efforts should be made to model the CYP1A1 - PCB relationship with a stepwise additive model that can directly test whether the addition of individual congeners improves the relationship between CYP1A1 and PCB concentrations. For data sets with sufficient numbers of samples and extensive individual congener analysis, this approach could indicate which PCBs are most likely linked to CYP1A1 induction. Comparison of the CYP1A1 levels and PCB congeners between different species would indicate whether the most important PCBs for CYP1A1 induction vary locally, or are similar globally. This research could be supplemented by binding studies of the

important PCB congeners, based on both total concentration and the model, to the aryl hydrocarbon receptor (AHR), to determine whether those congeners identified in the model have a higher bind affinity for the AHR. This approach would model initial studies of TCDD binding of the beluga AHR (Jensen and Hahn 2001).

For much of this work, dose-response relationships would prove invaluable for interpreting the extent of CYP1A1 induction in free-ranging animals. Since experimental work is precluded, primary cell cultures and precision slice cultures offer the best opportunities for determining the dose-response curve for CYP1A1 induction in cetacean cells. Although CYP1A1 has not been inducible in the available cell cultures (Appendix 3), with time and adequate screening, it may be possible to identify a responsive primary culture. Ongoing efforts to isolate and culture hepatocytes, where CYP1A1 expression is highest (Chapter 3), and responsive endothelial cells, the cell type with highest CYP1A1 expression in integument biopsies (Chapter 4) are extremely important for this aim.

Efforts to measure CYP1A1 in integument biopsies of free-ranging animals needs much work. We need to initiate studies to understand the role of mobilization and deposition and lipid content on CYP1A1 expression in endothelial cells of the dermis. Studies with animals which are fasting or lactating, when energy stores are often mobilized, would be useful in understanding what are the contributing factors to endothelial CYP1A1 expression in the dermis. Since most biopsy studies take place using remote sampling with cross-bows or adapted rifles, the sampling location on the body can vary (as in Appendix 2). The interpretation of such data requires an assumption that CYP1A1 expression does not vary across the body of an individual animal. However, data from a single right whale indicates that there were large differences in CYP1A1 expression, depending on where the sample on the body was taken (C. Miller, pers. com.). Systematic sampling of integument samples across the body would allow us to build a topographical model of CYP1A1 expression and identify regions where CYP1A1 is invariant in a single animal. If this information were coupled with lipid content, blubber thickness, and fatty acid content, we could perhaps better understand the physiological basis for CYP1A1 expression to vary in integument. Finally, the implications of CYP1A1 induction in integument for the health of the animal are unclear,

as it is a measurement in a single organ that has not traditionally been seen as important for PHAH toxicity. Much work is needed to understand the possible role of endothelial CYP1A1 in contaminant flux and the relationship between measures in integument for the whole animal.

Lastly, although this research identifies differences in sensitivity to CYP1A1 induction between cetacean species, we are interested in the risk of toxicity for these species. Completion of similar studies in beluga whale, bottlenose dolphin, and white-sided dolphin with other endpoints of toxicological interest would be of great use to compare relative sensitivity between CYP1A1 and other measures. Since one of the major effects of PHAH contaminants in marine mammal populations is immunotoxicity, studies that contrast concentrations that induce CYP1A1 with concentrations that cause immunotoxicity would be important. Studies such as the International Whaling Commission's Pollution 2000+ program (see chapter 4), where multiple endpoints of toxicological interest are examined in the same samples, could help elucidate which measures constitute useful biomarkers of effects in these species.

Cetacean CYP19 (Aromatase)

The study of CYP19, or aromatase, in cetaceans was chosen based on the importance of estradiol for reproduction and development and studies that suggested that aromatase can be inhibited by some PHAHs, including PCBs (Drenth *et al.* 1998; Hany *et al.* 1999; Gerstenberger *et al.* 2000). Successful reproduction is critical for survival of species, especially in endangered cetaceans. The goals of this research were to determine (i) the CYP19 gene sequence from a cetacean, (ii) the phylogeny of aromatase across the vertebrates and the evolutionarily diverse regions of the gene, and (iii) the functional characteristics of the cetacean CYP19.

Gene Sequence, Phylogenetics and Evolutionary Divergence

The studies of white-sided dolphin aromatase supported the current understanding that aromatase is a well conserved protein with high sequence similarity across diverse taxa (Chapter 5). The conservation of aromatase was shown in analyses of the amino acid sequence across diverse vertebrate taxa. Any divergence in aromatase between

species must be predominantly at a transcriptional level. This would suggest that the functional characteristics of the protein are also very similar across taxa.

Transient Transfections: an in vitro Measure of Functional Characteristics

In vitro assessment of functional characteristics of the dolphin aromatase indicated that this protein was very similar in function to other mammalian aromatases. The dolphin aromatase has a K_m and V_{max} for both testosterone and androstenedione similar to values for other mammalian aromatases. Transient and stable transfection systems have been used to express both human (Ackerman *et al.* 1981; Corbin *et al.* 1988; Pompon *et al.* 1989; Zhou *et al.* 1990; Stresser *et al.* 2000) and pig (Corbin *et al.* 1999) aromatases. The *in vitro* functional characterization of dolphin aromatase and the DIVERGE analyses both indicate that at least mammalian aromatases, and likely most vertebrate aromatases, have very similar functional characteristics. Thus, studies in rodent or other mammalian systems could apply as well to most mammalian species. Ongoing efforts to document the similarities in these systems will allow us to eventually understand where the similarities and differences exist in the control of aromatase expression and expression patterns between mammalian systems.

Future Work with Aromatase in Cetaceans

Both the DIVERGE analysis and the transient transfection work demonstrated that this protein is well conserved across vertebrate taxa. It appears that research in other mammalian species should be applicable to cetaceans. However, aromatase, contrary to CYP1A1, has not been well studied in many mammalian species, which makes it difficult to know where similarities and differences exist beyond the functional characterization of kinetic properties, and expected expression in ovary. Placental expression of aromatase has not been detected in all mammals studied, but has been identified in primates and the artiodactyls (i.e. cow, pig, sheep) tested to date (Hinshelwood *et al.* 1997; Graddy *et al.* 2000; Conley and Hinshelwood 2001; Vanselow *et al.* 2001). Although we might expect placental expression in cetaceans, based on their phylogenetic position, only a few species have been tested within the artiodactyls. Widespread expression in multiple internal organs has been clearly demonstrated in humans. With the recent evidence from the ArKO mouse models (Fisher *et al.* 1998; Honda *et al.* 1998), it would appear that

aromatase has an important role, as expected based on knowledge of estradiol function in development, reproduction, and normal physiology. Whether aromatase has widespread expression or more limited expression has yet to be clearly determined in multiple species. Ongoing work on promoter regions (Hinshelwood *et al.* 1997; Callard *et al.* 2001; Vanselow *et al.* 2001), control of aromatase expression by nuclear receptor and signaling molecules (Brueggemeier *et al.* 2001; Shozu *et al.* 2001; Yanase *et al.* 2001; Catalano *et al.* 2003; Enjuanes *et al.* 2003), and effects of contaminants (Drenth *et al.* 1998; Hany *et al.* 1999; Letcher *et al.* 1999; Dasmahapatra *et al.* 2000; Gerstenberger *et al.* 2000; Moran *et al.* 2000; Sanderson *et al.* 2001) should provide important information that will need to be verified and contrasted between diverse species. Most research on aromatase is in human, cow, pig, and mouse, but application of this research to wildlife species will require much effort. This work, and recent studies in other wildlife species such as possum (Whale *et al.* 2003), will aid in understanding the similarities in aromatase expression and function across diverse species.

Of primary importance for cetaceans is documentation of the sites of expression in various internal organs. Certainly, some organs, such as placenta, brain, and gonads are most likely to express aromatase. Some organs, such as brain, will be very difficult to sample in cetacean species. Obtaining brain samples has proved difficult because of the difficulty of gaining access to the brain case in the field and the speed with which brain degrades compared to other organs. Since aromatase is expressed in fat stores and the ArKO mouse has increased internal fat stores (Jones *et al.* 2000; Jones *et al.* 2001; Toda *et al.* 2001), it would be very interesting to examine aromatase expression in blubber of cetaceans. More interesting is the discovery of a regulatory role of leptin, the so-called obese gene, in controlling aromatase expression in the MCF-7 breast cancer cell line (Catalano *et al.* 2003). Leptin plays a critical role in fat storage (reviewed in Mohamed-Ali *et al.* 1998). Since accumulation of significant fat stores appears to coincide with successful reproduction (Lockyer 1986), studies on leptin and aromatase in blubber could aid in understanding physiological factors controlling reproduction in cetaceans.

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Appendix 1. Cytochrome P450 1A1 expression in cetacean integument: implications for detecting contaminant exposure and effects

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Abstract

Contaminant related health risks to marine mammals are typically inferred from the levels of contaminants measured in blubber. Such measurements alone are insufficient to indicate the likelihood of biological effects from contaminant exposure, especially for contaminants that do not bioaccumulate. Cytochrome P450 1A1 (CYP1A1) in mammals is induced by and involved in the metabolism of planar halogenated aromatic hydrocarbons (PHAHs) and polycyclic aromatic hydrocarbons (PAHs), chemicals of concern in aquatic systems. CYP1A induction is a molecular effect of exposure to these inducers in many vertebrates. Here we summarize studies of CYP1A1 expression detected immunohistochemically in integument (epidermis and blubber) collected by biopsy or at necropsy from 17 species of cetaceans. CYP1A1 expression was detected in all species, but varied both within and between species. CYP1A1 levels in mysticetes were generally similar to those in odontocetes. Assessing how differences between individuals, populations or species affect CYP1A1 expression in cetacean integument is essential to the interpretation of this induction as a biomarker of exposure to and effects of contaminants. Detection of CYP1A1 expression in integument samples offers a simple, non-lethal technique to study biological changes associated with contaminant exposure in cetaceans.

Introduction

Marine mammals have long been known to accumulate lipophilic contaminants such as halogenated aromatic hydrocarbons (HAHs) (*e.g.*, polychlorinated biphenyls (PCBs)) and pesticides (*e.g.*, dichlorodiphenyltrichloroethane (DDT)) in their blubber. Mysticetes typically have lower concentrations of these contaminants than odontocetes, presumably the result of feeding at a lower trophic level (O'Shea and Brownell 1994). PCB concentrations as high as 200 to 300 μg/g lipid weight have been recorded in odontocete blubber (Aguilar and Borrell 1994; Ross *et al.* 2000). At these concentrations, the PCBs in a mere 18.6g of blubber would be equivalent to the LD50 (dose causing 50%

mortality) for PCBs in rat[1]. Such concentrations of contaminants have raised concerns that marine mammal populations may be at risk to contaminant induced effects. However, in the absence of explicit dose-response information, contaminant concentrations alone are not sufficient to estimate risk to individuals or populations, especially for contaminants that do not bioaccumulate, such as polycyclic aromatic hydrocarbons (PAH). Measurement of biological changes associated with contaminant exposure can provide information critical to assessing the impacts of contaminants in marine mammals.

Several studies have indicated that PCB toxicity may be occurring in marine mammal populations. Epizootic outbreaks of morbillivirus and phocine distemper virus have been associated with high PCB concentrations in populations of striped dolphin in the Mediterranean Sea (Aguilar and Borrell 1994) and harbor seals in the Baltic Sea (Hall *et al.* 1992). Experimental studies have demonstrated a wide range of immunological impacts associated with consuming PCB tainted fish in harbor seals (De Swart *et al.* 1996; Ross *et al.* 1996), supporting the hypothesis that PCBs are immunotoxic agents in marine mammals. Reproductive difficulties in harbor seals also have been associated with high PCB loads (Reijnders 1986). The highly contaminated beluga whales in the St. Lawrence estuary (a population of approximately 650 animals) suffer from 39% of all cancer cases reported in captive and wild cetaceans worldwide and have cancer rates (adjusted annual rate of 507/100,000 animals for all cancer types) higher than that observed in other wildlife and domesticated animal species and higher than that observed in humans (Martineau et al. 2002). As well there are multi-systemic and viral lesions in the St. Lawrence beluga whales not observed in other cetacean populations (Martineau *et al.* 1988). Such associations between high contaminant loads and population health

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[1] The average LD50 for Aroclor mixtures [1254, 1260, 1221, 1232, 1242] (NTP reports, www.niehs.nih.gov) is 3005 mg/kg. The PCB concentrations in killer whale blubber (254.7 mg/kg lipid weight) were converted to wet weight (161.5mg/kg wet weight) based on an average lipid content of 63.4% in killer whale blubber (Ross *et al.* 2000). Thus, a "dose" of 18.6g blubber/g rat body weight is equivalent to the LD50 for PCBs in rat.

problems emphasize the need to measure early biological changes of contaminant exposure.

Members of the cytochrome P450 1A gene subfamily in mammals are inducible by planar HAHs (PHAHs) (*e.g.*, non-*ortho* substituted PCB congeners and 2,3,7,8-tetrachlorodibenzo-p-dioxin) and by PAHs (*e.g.*, benzo[a]pyrene) that are ligands for and activate the aryl hydrocarbon receptor (AHR). Two cytochrome P450 1A genes have been detected in mammals, cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1A2 (CYP1A2). CYP1A1 has been detected in cetaceans by immunoassays (White *et al.* 1994) and cDNA sequencing (Teramitsu *et al.* 2000) while the paralogous CYP1A2 has not. As yet, there is no evidence to support multiple CYP1A genes in cetaceans and based on the specificity of the antibody used in this study, we refer to the protein that we detect in cetaceans as CYP1A1. CYP1As are involved in the metabolism of most inducers (Whitlock 1999) and cetacean CYP1A1 appears to oxidize PAHs and non-*ortho* PCBs (White *et al.* 1994; White *et al.* 2000). Induction of CYP1A1 can occur within hours of exposure and when inducing chemicals are cleared from the body, levels of CYP1A1 decrease. CYP1A1 induction can therefore be considered an early biological effect of exposure to contaminant inducers. Significantly, in rodents, CYP1A1 expression has been correlated to higher order toxic effects including thymic atrophy, weight loss and toxicity induced by PCB, PCDD and PCDF exposure (Poland and Knutson 1982; Safe 1987; Safe 1990). CYP1As can activate PAH protoxicants and, when bound with non-*ortho* PCBs, can generate reactive oxygen (Schlezinger *et al.* 1999), suggesting linkage of CYP1As to toxicity of these AHR agonists.

CYP1A induction has been used as a biomarker of exposure to PHAHs and PAHs in a wide variety of vertebrate species, including mammals (White et al. 1994; Qualls et al. 1998), fish (Smolowitz *et al.* 1991; Woodin *et al.* 1997), birds (Sanderson *et al.* 1994), reptiles (Bishop *et al.* 1998; Rie *et al.* 2000) and amphibians (Huang *et al.* 2001). In cetaceans, levels of CYP1A1 expression in liver of beluga whale were correlated to concentrations of Σ mono-*ortho* and non-*ortho* PCBs (known inducers of CYP1A1) in blubber (White *et al.* 1994), and elevated skin benzo[a]pyrene monooxygenase activity (which is attributable to CYP1A1 expression) was correlated with higher organochlorine

concentrations in blubber of fin whale and striped dolphin (Fossi *et al.* 1992). These studies indicate that in cetaceans the expression of CYP1A1 in skin, as well as in internal organs, can relate to levels of contaminants in blubber.

To study PHAH and PAH induced effects in marine mammals, we developed and have been validating the use of immunohistochemistry for detection of CYP1A1 induction. For the past 12 years, we have applied this technique to the analysis of integument of a wide variety of cetacean species. A summary of results of this work and the variables needing consideration for interpretation of CYP1A1 expression data from cetacean integument are discussed herein.

Methods

Integument samples (epidermis and underlying blubber) were collected by biopsy or at necropsy from free-ranging, by-caught, native subsistence hunted, stranded or aquarium cetaceans. Species and locations sampled are listed in Table 1.

CYP1A1 expression was examined using immunohistochemistry, as previously described for fish (Smolowitz *et al.* 1991). Briefly, the samples (approximately 0.5g tissue) were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 5μm. Serial sections were deparaffinated, hydrated and stained immunohistochemically using a peroxidase anti-peroxidase detection system (Signet Laboratories, Dedham, MA) with the monoclonal antibody (MAb) 1-12-3 to scup (*Stenotomus chrysops*) CYP1A as the primary antibody and non-specific MAb MOPC or UPC-10 (Sigma-Aldrich Co., St. Louis, MO) as the control antibody. MAb 1-12-3 recognizes an epitope that occurs in CYP1A1 but not CYP1A2 in mammals (Drahushuk *et al.* 1998), and recognition by this antibody thus reflects a probable CYP1A1 in cetaceans. Amino-9-ethylcarbazole (AEC; Signet Laboratories, Dedham, MA) was used as the chromogenic substrate for visualization of CYP1A1. Sections were counterstained with Mayer's hematoxylin (Sigma Diagnostics, St. Louis, MO) for visualization of nuclei. Stained sections were evaluated under light microscopy for stain occurrence (scale of 0-3) and stain intensity (scale of 0-5) in each cell type. CYP1A1 expression was calculated as the product of the stain occurrence and intensity to generate a semi-quantitative index

(scale of 0-15). A linear relationship between this staining index and CYP1A protein content detected by quantitative immunoblot assay has been shown (Woodin *et al.* 1997).

Results and Discussion

To date we have examined CYP1A1 expression in integument samples from 6 mysticete and 11 odontocete species (Table 1). In all 17 species, CYP1A1 expression was strongest and occurred most often in vascular endothelial cells of the arterial system and of capillaries (Figure 1) consistent with our earlier observations that CYP1A is highly inducible in endothelium of vertebrates (Stegeman et al. 1989). Table 1 shows the average levels of endothelial CYP1A1 staining index in integument from the various species and from different geographical locations. These values ranged from 0 (n=2) in killer whale (British Columbia, Canada) to 8 ± 3 (n=5) in Risso's dolphin (Cape Cod, USA). CYP1A1 staining was, at times, detected also in vascular smooth muscle, macrophages, fibroblasts and peripheral nerves but not in elastin fibers, collagen fibers, adipose tissue, epidermis or venular endothelia.

The extent of CYP1A1 induction in vascular endothelial cells in cetacean integument *in vivo* is likely to be related to concentrations of AHR agonists in the blood. First, in mammalian endothelial cells in culture, CYP1A1 is induced in a dose dependant way by various AHR agonists (Stegeman *et al.* 1995). Secondly, the induction of CYP1A1 in sperm whale integument *in vitro* by β-napthoflavone (βNF, a prototypical CYP1A1 inducer) occurs in a dose dependent way (C. Godard, personal communication). Thirdly, in captive river otters experimentally fed crude oil, a dose-response relationship was observed between hydrocarbon content in the diet and CYP1A1 levels detected by immunohistochemistry in the vascular endothelia of skin biopsies (Ben-David *et al.* 2001). As previously mentioned, CYP1A1 expression in cetacean skin and liver was related to contaminant levels in the blubber (Fossi *et al.* 1992; White *et al.* 1994).

It is evident from Table 1 that the level of CYP1A1 staining in vascular endothelia of cetacean integument varies both within and between species. The range of CYP1A1 staining in mysticetes was similar to that in odontocetes. This is intriguing, given that mysticetes typically have lower levels of organochlorines in blubber (O'Shea and

Brownell 1994). It is also intriguing that the level of CYP1A1 expression in the killer whales was extremely low, while PCB concentrations were quite high in killer whale blubber from the same area where our samples were taken (Ross *et al.* 2000). Therefore, factors in addition to tissue burdens of persistent organohalogens must influence CYP1A1 expression in cetacean integument and supplementary data are needed to extend the interpretation of CYP1A1 expression scores. A number of variables possible affecting CYP1A1 expression are considered below.

AHR agonists in blood could derive from recent diet or from residues in the lipid-rich blubber, and in a given species, blood and tissue concentrations of AHR agonists could differ with geographic location, trophic level, gender and age, thereby potentially affecting CYP1A1 expression. The sources of contaminants to a food chain or habitat can vary with geographic location. Proximity to major sources of contaminants should lead to higher levels of contaminant exposure. Animals feeding at higher trophic levels can be exposed to higher contaminant concentrations due to accumulation of persistent contaminants in the tissues of the prey. Persistent contaminants also can accumulate in the tissues of cetaceans over time; older animals tend to have higher contaminant concentrations in blubber (Aguilar and Borrell 1988). This is especially true for males rather than females, as females offload persistent contaminants to offspring during gestation and lactation (Aguilar and Borrell 1988).

CYP1A1 expression in vascular endothelia in cetacean integument could be affected also by movement of AHR agonists across the endothelial cells from the blood to the tissue or vice versa (*i.e.*, *via* mobilization or deposition of lipids containing lipophilic contaminants). Blubber not only varies in thickness and composition across the body of a cetacean, but also can vary in lipid composition with depth from the epidermal surface, demonstrating that blubber is more metabolically active in some parts of the animal (Ackman et al. 1975; Lockyer et al. 1985; Aguilar and Borrell 1990; Koopman et al. 1996; Koopman 1998; Koopman et al. 2002). Migration status, reproductive status (especially of reproducing females), and food availability can affect the energy state of the animal, thereby potentially increasing mobilization or deposition of lipids. If lipophilic contaminants in blubber are similarly mobilized at the areas of increased

metabolic activity within the blubber, CYP1A1 induction could also be affected. Therefore, integument samples from different locations along the body of an individual cetacean and histological sections from different depths in the biopsy could differ in the amount of CYP1A1 expression, a possibility supported by preliminary results from our studies (J. Wilson, unpublished data). However, given random sampling of groups of individuals, it is reasonable to make statistical inferences at the group level, as has been shown with fish (Woodin et al. 1997) and birds (Sanderson et al. 1994).

The dose-response and structure-activity relationships for induction of CYP1A1 in cetacean species have not been defined. Species are known to vary widely, more than 5000-fold, in the responsiveness to inducers of CYP1A (Poland and Knutson 1982; Hahn 1998). Thus, dose-response data are needed to define the specific response of a given species. Likewise, CYP1A inducers can differ in potency between species (Poland and Knutson 1982; Hahn 1998): potency can be inferred from agonist affinity for the AHR (Safe 1990; Hestermann *et al.* 2000; Jensen and Hahn 2001), but coupling of an activated receptor to a response can vary between cell types. It is important to establish the responsiveness of vascular endothelial cells to CYP1A inducers in multiple species.

Our analyses to date indicate that CYP1A1 is induced in peripheral microvasculature of many cetacean species. The location of the sample along the body, the depth of the sample, the sampling season and/or the sampling year, the geographic location of the animal when sampled and the species, gender, age class, and energetic state of the animal are all variables that could influence CYP1A1 expression in cetacean integument. It will take careful consideration of each of the above variables to properly interpret integument CYP1A1 expression data in cetaceans but based on studies in other wildlife species comparisons between randomly sampled groups remain valid, in spite of potential individual variability. We are engaged in studies to assess the contribution of these variables to better interpret this molecular change as related to exposure of the individual.

The measurement of CYP1A1 levels, or changes in expression of other genes, in integument biopsies offers a unique approach to study biological changes associated with both persistent and non-persistent contaminants in cetaceans, using a system that is well

described in many other species. We have emphasized immunohistochemical methods as opposed to measuring catalytic activity, western blot, or measurement of mRNA. While these methods are feasible, the advantages of the immunohistochemical approach are: (a) ease of use for field and shipboard research; (b) the need for only small amounts of tissue; (c) preservation of tissue samples in fixative until transport back to the laboratory for processing; (d) storage of fixed tissue samples at room temperature. The relationship between CYP1A1 expression in the integument and that in internal organs is being defined. Such relationships should enhance our ability to interpret CYP1A1 levels in biopsies in terms of the significance to the health of the organisms, as well as to exposure to a subset of toxic chemicals in the global environment.

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Table 1. Vascular endothelial cytochrome P450 1A expression [Mean ± SD (N)] in cetacean integument. [1]Stranding, [2]Biopsy, [3]Aquarium, [4]Native Hunt, [5]By-Catch, [6]Semiquantitative staining index (0-15) in vascular endothelium of the integument. Samples were obtained with the most generous assistance of colleagues listed in the acknowledgements.

Species	Common Name	Year	Site	CYP1A[6]
Mysticetes				
Eubalaena glacialis	Northern Right Whale	1993 to 1997	Bay of Fundy [2]	3.8 ± 2.5 (83)
Eubalaena glacialis	Northern Right Whale	1996 to 2000	Cape Cod Bay [2]	2.5± 1.5 (17)
Eubalaena glacialis	Northern Right Whale	1997 to 1998	Georgia USA [1,2]	0.3 ± 1.0 (9)
Eubalaena australis	Southern Right Whale	1995 to 2000	Valdez, Argentina [1]	1.3 ± 1.8 (12)
Eubalaena australis	Southern Right Whale	1997	South Georgia [2]	1.7 ± 1.5 (3)
Eubalaena australis	Southern Right Whale	1996, 1997	South Africa [2]	2.2 ± 1.6 (121)
Balaenoptera acutorostrata	Minke Whale	1991	Cape Cod, MA[1]	2.0 (1)
Balaenoptera musculus	Blue Whale	1996 to 2000	Gulf of St Lawrence [2]	4.7 ± 1.9 (48)
Balaenoptera musculus	Blue Whale	1997, 2000	Iceland [2]	2.2 ± 1.9 (12)
Balaenoptera physalus	Fin Whale	1994	Mediterranean [2]	1.5 ± 2.1 (2)
Balaenoptera physalus	Fin Whale	1998 to 2000	Gulf of St Lawrence [2]	3.1 ± 1.7 (14)
Balaenoptera physalus	Fin Whale	1997	Stellwagen Bank, MA[2]	4.0 (1)
Megaptera novaeangliae	Humpback Whale	1991, 1993, 1997	Stellwagen Bank, MA[2]	3.4 ± 2.7 (32)
Megaptera novaeangliae	Humpback Whale	1994	Virginia [2]	4.3 ± 0.4 (2)
Megaptera novaeangliae	Humpback Whale	1998 to 2000	Gulf of St Lawrence [2]	2.5 ± 2.5 (13)

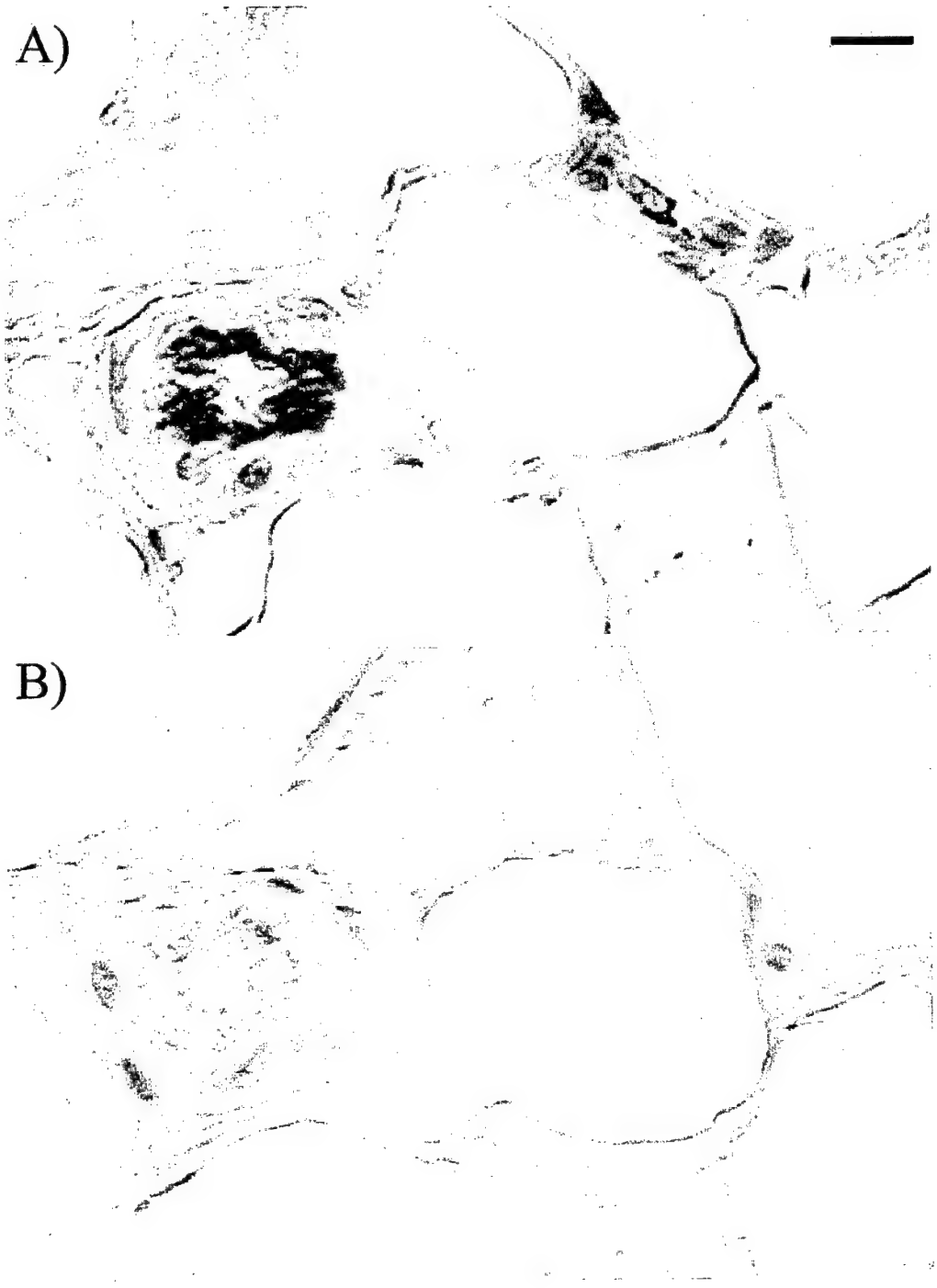
Table 1 Continued

Species	Common Name	Year	Site	CYP1A[6]
Odontocetes				
Delphinapterus leucas	Beluga Whale	1996	Mackenzie Delta, Canada[4]	5.2 ± 1.6 (13)
Delphinapterus leucas	Beluga Whale	1994	Tacoma, WA[3]	4.0 (1)
Delphinapterus leucas	Beluga Whale	1994	Tacoma, WA[3]	0.0 (3)
Delphinus delphis	Common Dolphin	1997	Cape Cod, MA[1]	5.6 ± 2.2 (6)
Delphinus delphis	Common Dolphin	1998	Western Atlantic[2]	5.4 ± 2.9 (82)
Delphinus delphis	Common Dolphin	1994	Mediterranean[2]	3.0 ± 3.0 (3)
Delphinus delphis	Common Dolphin	1996	Northwest Atlantic[5]	2.7 ± 2.6 (4)
Globicephala melas	Pilot Whale	1991	Cape Cod, MA[1]	3.1 ± 2.5 (13)
Globicephala melas	Pilot Whale	1996	Northwest Atlantic[5]	0.3 ± 0.7 (4)
Grampus griseus	Risso's Dolphin	1994	Cape Cod, MA[1]	8.1 ± 3.0 (5)
Hyperoodon ampullatus	Bottlenose Whale	1996, 1997	Nova Scotia, Canada[2]	0.2 ± 0.7 (19)
Lagenorhynchus acutus	White-sided Dolphin	1994	Cape Cod, MA[1]	8.0 (1)
Mesoplodon bidens	Sowerby's Beaked Whale	1996	Northwest Atlantic[5]	1.8 ± 1.8 (3)
Orcinus orca	Killer Whale	1994	Prince William Sound[2]	0.4 ± 0.8 (21)
Orcinus orca	Killer Whale	1993	British Columbia, Canada[2]	0.0 ± 0.0 (2)
Phocoena phocoena	Harbor Porpoise	1994	Tacoma, WA[3]	4.5 (1)
Phocoena phocoena	Harbor Porpoise	1994	Grand Manan, NB, Canada[1,5]	0.1 ± 0.17 (9)
Phocoena phocoena	Harbor Porpoise	1996	Cape Cod, MA[1]	0.0 ± 0.0 (2)
Physeter macrocephalus	Sperm Whale	1999	Sea of Cortez[2]	3.6 ± 1.2 (13)

Table 1 Continued

Species	Common Name	Year	Site	CYP1A[6]
Physeter macrocephalus	Sperm Whale	1998	Georges Bank [2]	1.0 ± 1.4 (2)
Physeter macrocephalus	Sperm Whale	1994, 1995	Galapagos [2]	0.6 ± 1.1 (9)
Tursiops truncatus	Bottlenose Dolphin	1995	San Diego, CA [2,3]	5.0 ± 0 (2)
Tursiops truncatus	Bottlenose Dolphin	1994	Gulf of Mexico [1]	4.7 ± 2.7 (6)
Tursiops truncatus	Bottlenose Dolphin	1998	Western Atlantic [2]	3.6 ± 2.6 (141)
Tursiops truncatus	Bottlenose Dolphin	1994	Mediterranean [2]	3.5 ± 0.7 (2)

Figure 1. Immunohistochemical localization of cytochrome P450 1A1 (CYP1A1) in vascular endothelium of two small arterioles in common dolphin (*Delphinus delphis*) blubber. A) High CYP1A1 expression is visualized by the strong red stain. B) Serial section of sample in (A) stained with non-specific control MAb MOPC. Sections were counterstained with Mayer's hematoxylin. Scale bar represents 10 μm.


A)
B)

Appendix 2. Expression of Cytochrome P450 1A1 in Integument Biopsies from Offshore Free-ranging Bottlenose (*Tursiops truncatus*) and Common (*Delphinus delphis*) Dolphin

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Abstract

The expression of cytochrome P450 1A1 (CYP1A1) in integument biopsies of cetacean species appears to vary with the geographic area of the biopsied animal (Appendix1, Miller *et al.* Submitted). Integument biopsies were collected from free-ranging bottlenose and common dolphin in offshore waters of the western Atlantic Ocean, between Georges Bank and Cape Hatteras NC. CYP1A1 expression was determined immunohistochemically, to identify if regional variation in the expression of CYP1A1 within a species could be identified within a small segment of an ocean basin. CYP1A1 expression was similar between most cell types that expressed the protein, and between both species. CYP1A1 expression, in both species, varied within the dermis of a given biopsy and endothelial cells in the lower dermis expressed higher CYP1A1, as had been seen in other bottlenose dolphin samples (Chapter 4). CYP1A1 expression in endothelial cells of the lower dermis appeared to be correlated with the relative amount of adipocytes in bottlenose dolphin. A similar analysis was not performed with common dolphin. Geographical variation in CYP1A1 expression was addressed by comparing CYP1A1 expression and latitude and longitude. No simple relationship between these variables existed and no variation was seen in the common dolphin samples. More complex geographical variation in CYP1A1 expression from bottlenose dolphin seemed evident from comparisons of CYP1A1 expression along latitudinal and longitudinal gradients or by grouping samples into broad regions. Bottlenose dolphin, collected offshore of Cape Hatteras, had higher CYP1A1 expression than those collected at some other locations. When contaminant concentrations are available from these samples, the reasons for this geographical difference may be better understood.

Introduction

For many studies of cetaceans, tissue samples are required. Tissue is most often collected from stranded animals, although the health of these animals is often in question. Tissues collected during native subsistence hunts and from animals by-caught in fisheries are presumed to be healthy animals more representative of the population. Yet, tissues

cannot be collected by these means for all cetacean species. Integument biopsies allow for the collection of a small piece of epidermis and underlying blubber (skin, skin and blubber) from free-ranging animals. The biopsy of cetaceans has been shown to have minimal impact on the animals and wound healing appears to occur rather quickly in bottlenose dolphin (Krutzen *et al.* 2002; Parsons 2003). Integument biopsies have been used for determination of contaminant concentrations and as a source of RNA and DNA for studies of population genetics. CYP1A1 is expressed in several cell types present in integument in multiple species of cetaceans (Miller *et al.* Submitted). CYP1A1 activity has been measured in biopsies from several cetacean species (Fossi *et al.* 1992; Marsili *et al.* 1998; Fossi *et al.* 2000) and correlates to organochlorine concentrations in blubber (Chapter 4, Fossi *et al.* 1992; Marsili *et al.* 1998). CYP1A1 expression in endothelial cells, the cells that line the blood vessels and capillaries, varies within a species depending on the geographical location of the animal (Miller *et al.* Submitted). This variation is presumed to be due to different exposure levels between geographic locations, however this has not been directly tested.

Bottlenose dolphin and common dolphin integument biopsies were taken from primarily offshore animals in the western Atlantic Ocean in order to understand the impact of geographic variation on CYP1A1 expression in integument. Contaminant concentrations are not currently available for the animals in this study. Variation in CYP1A1 expression in animals from different locations would suggest that there were differences in contaminant exposure. Since the animals in this study cover a large region, this study may indicate the spatial scales needed to see differences in CYP1A1 expression in a single species. Furthermore, this study gives us the opportunity to compare the levels of CYP1A1 in two species that have similar life-history and overlapping habitats.

Materials and Methods

Integument samples (epidermis and underlying dermis or blubber) were collected from free-ranging bottlenose (*Tursiops truncatus*) and common (*Delphinus delphis*) dolphin in the western Atlantic Ocean, along the US east coast. A total of 147 bottlenose and 99 common dolphin biopsies were collected from a geographic area ranging from

Georges Bank to Cape Hatteras, NC. Biopsies were taken with a Barnett 'Wildcat' crossbow and Larsen biopsy tips (5 mm internal, variable length, Palsboell *et al.* 1991) from either the deck of the Delaware II (≈10 m above sea level) or from a zodiac. Information was collected on group size, species and geographic location for each group of animals encountered. For each biopsy attempt, a record of the reaction was taken. Photographs and video were taken for species confirmation, whenever possible. Since individual animals were unknown, a small proportion of each group was biopsied to prevent replicate sampling of the same animal. Age and sex were unknown in these animals although a notation of size (small, medium, or large) was taken for each animal.

The dorso-lateral aspect near the dorsal fin was the preferred biopsy location. Biopsy samples varied in depth, depending on the penetration of the biopsy tip and the angle of contact between the biopsy tip and the animal. Biopsy tips containing sample were wrapped in clean foil, placed in a whirl-pac bag and kept on ice until further processing. Biopsy tips were scrubbed cleaned in ethanol and rinsed with distilled, deionized water prior to reuse. Biopsy samples were processed within 1 hour of sampling. Samples were subdivided, depending on the size of the biopsy. For this study, a full depth, thin (≤ 3 mm) section was placed in 10% neutral buffered formalin and stored at room temperature.

Immunohistochemical analysis of CYP1A1 expression

CYP1A1 expression was determined in individual cell types present in integument using immunohistochemistry, as previously described for fish (Smolowitz *et al.* 1991). Briefly, the formalin-fixed samples were embedded in paraffin and sectioned at 5μm. Sections were deparaffinated, hydrated and stained immunohistochemically using a peroxidase anti-peroxidase detection system (Signet Laboratories, Dedham, MA) with the monoclonal antibody 1-12-3 to scup (*Stenotomus chrysops*) CYP1A as the primary antibody. This antibody recognizes an epitope specific to CYP1A1 in mammals and detects CYP1A in taxonomically diverse vertebrates including cetaceans (Stegeman and Hahn 1994). Serial sections of each biopsy were stained immunohistochemically with a negative control antibody, MOPC31 (Sigma, St. Louis MO), as the primary antibody. Amino-9-ethylcarbazole (AEC) was used as the chromogenic substrate for

visualization of CYP1A1. Nuclei were made visible by hematoxylin counterstaining. Stained sections were evaluated under light microscopy for stain occurrence (scale of 0-3) and stain intensity (scale of 0-5) in each cell type. CYP1A1 expression was calculated as the product of the stain occurrence and intensity to generate a semi-quantitative index (scale of 0-15). A linear relationship between this staining index and CYP1A protein content detected by immunoblot was shown previously (Woodin *et al.* 1997).

For bottlenose dolphin biopsies, estimates of the relative percent of adipocytes in the lower dermis were made by visual inspection. Estimates were made on serial sections stained with hemotoxylin and eosin, and were scored based on multiple tissue slices.

Results

For both species of dolphins, the majority of samples (>80%) were from large, adult animals (Table 1). Biopsies were taken from common dolphins over a geographic area ranging from 37.45° to 40.09° latitude and 68.35° to 74.26° longitude. The bottlenose dolphin samples were taken over a larger geographic region, from 34.43° to 40.56° latitude and 67.07° to 75.66° longitude. Sex was not known for nay of the animals.

CYP1A1 expression was seen in vascular endothelial cells, vascular smooth muscle and peripheral nerves in both species. Neither bottlenose nor common dolphin expressed CYP1A1 in connective tissue, epidermis, or adipocytes. A few common dolphin, but no bottlenose dolphin, had slight CYP1A1 expression in the perineurium, the cells that surround the nerve bundle. Average CYP1A1 staining in these cell types are shown in Table 2. Mean CYP1A1 staining is very similar amongst all cell types, except endothelial cell of the upper dermis. There are no differences in CYP1A1 staining between the two species studied, for any cell type. Expression of CYP1A1 in endothelial cells of the lower dermis correlated with endothelial expression in the upper dermis ($r=0.33$, $p<0.05$) and in nerve cells ($r=0.32$, $p<0.05$) in bottlenose dolphin (data not shown). Endothelial expression of CYP1A1 in the lower dermis was also correlated with nerve cells in common dolphin ($r=0.37$, $p<0.05$, data not shown). There were no significant correlations in CYP1A1 expression between any other cell types. There is no

difference in CYP1A1 expression, in any cell type, when animals are grouped by age or size class, for either bottlenose dolphin or common dolphin.

Endothelial CYP1A1 expression varied within the dermis of a biopsy. Endothelial cells in the region immediately next to the epidermis, at the dermal – epidermal interface, had lower CYP1A1 expression than endothelial cells deeper in the dermis (denoted the lower dermis). This variation in endothelial cell CYP1A1 expression was present in both species (Table 2). Endothelial CYP1A1 expression was correlated with percent adipocytes in the lower dermis ($r=0.45$, $p<0.05$, data not shown) in bottlenose dolphin. A similar analysis was not performed with the common dolphin samples.

Since genetics analysis and stock assessment is incomplete, it is difficult to divide these samples into geographic stocks. During the field sampling, animals were biopsied in five broad regions (from north to south): Georges Bank, Southern New England, Mid-Atlantic, Southern Mid-Atlantic, and Cape Hatteras. All biopsies were coded, during the cruise, for the geographic region where they were collected. Common dolphins were not sampled in the Cape Hatteras region. There was no difference between CYP1A1 expression in endothelial cells of the lower dermis, when samples were divided into these regions, except for the bottlenose dolphin samples from the Cape Hatteras region, which had significantly higher CYP1A1 expression (data not shown) than the animals from all other regions.

There was a correlation between latitude and CYP1A1 expression in nerve cells in common dolphin ($r=0.40$, $p<0.05$) and endothelial CYP1A1 expression of the lower dermis in bottlenose dolphin ($r=-0.41$, $p<0.05$). Longitude did not correlate with any CYP1A1 score, in either species. Samples were grouped in one degree increments, based on both the latitude and longitude of the animal when sampled. CYP1A1 expression in lower endothelial cells did not vary across either latitude (Figure 1) or longitude in common dolphin (Figure 2). For bottlenose dolphin, CYP1A1 expression in lower endothelial cells did vary geographically (Figures 1 and 2). Samples had decreasing CYP1A1 expression from 35° to 40° latitude, before increasing again at 41° latitude. The mean CYP1A1 expression in samples taken at 35° latitude was significantly different

from the mean expression in samples taken from 38° to 41° latitude, however no other differences were statistically significant. Comparing bottlenose dolphin along a longitudal gradient indicated possible regional variation, as samples taken at 72° longitude had the lowest endothelial CYP1A1 expression in the lower dermis, and samples at 69° and 76° longitude had the highest endothelial CYP1A1 scores. Statistical analyses of these data indicated that only samples from 74° and 76° longitude were significantly different ($p<0.05$).

Discussion

The cell types that expressed CYP1A1 in integument samples from bottlenose and common dolphin are similar to that reported for other cetacean species (Miller *et al.* Submitted). CYP1A1 expression in the perineurium, the cells that surround the nerve bundle, has not been reported before, but this expression was very low and only present in a few common dolphins. There was little difference between CYP1A1 scores in most cell types, excluding endothelial cells of the upper dermis. This is an unusual finding as endothelial CYP1A1 expression has typically the highest CYP1A1 expression (Miller *et al.* Submitted). The high CYP1A1 expression in nerve cells has not been seen before, and the reasons for such expression and the biological implications of this expression are unknown. This type of high level expression was not seen in a study of CYP1A1 expression in integument from coastal bottlenose dolphin (Chapter 4). Correlations in CYP1A1 expression between the various cell types were usually lacking, although this was seen in another study of bottlenose dolphin biopsies (Chapter 4). CYP1A1 expression in endothelial cells of the lower dermis correlated with expression in nerve cells in both species. Although CYP1A1 expression was not different, in any cell type, when grouped by age or size class in either species, most of the samples were taken from large, adult animals. The power to distinguish differences in the other classes would have been small.

In bottlenose dolphin, estimates of percent of adipocytes in the lower dermis were determined by visual inspection of hemotoxylin and eosin stained sections. These data were not collected in common dolphin because the methodology is inexact, prone to bias,

and is not quantitative. Yet, for bottlenose dolphin, there is a correlation between the adipocyte content and CYP1A1 expression in endothelial cells of the lower dermis. The reasons for this relationship between adipose content and CYP1A1 expression in this cell type is unknown, however suggestions have been made that it could be related to lipid content, nutritional status, or mobilization and deposition of fat from adipocytes (Chapter 4). Quantitative measures of adipocyte content are difficult and there is no easy way to currently quantify the adipose content of these biopsy samples.

Without knowledge of the genetics of these offshore populations of dolphins, it is difficult to assign samples into reasonable groupings. With proper stock assessment, we could group animals that were in the same stock, based on known ranges of each stock. However, stock assessments have not been made for offshore populations of either common or bottlenose dolphin. Instead, geographic variation in the expression of CYP1A1 was assessed in three ways to try to identify if geographic variation in the expression of CYP1A1 existed. Simple regression of endothelial CYP1A1 expression in the lower dermis and either latitude or longitude identified a latitudinal gradient for bottlenose dolphin but not common dolphin. No gradient was seen for longitude, which indicates that either longitude has no influence on CYP1A1 expression or that there was a more complex relationship between longitude and CYP1A1 levels. Initial work during the cruise identified five major regions of the western Atlantic Ocean from which animals were sampled: Georges Bank, Southern New England, Mid-Atlantic, Southern Mid-Atlantic, and Cape Hatteras. After, dividing samples according to these regions, bottlenose dolphin from the Cape Hatteras region had significantly greater endothelial CYP1A1 expression, in lower dermis, than any other region.

Division of samples into one degree increments also showed interesting trends and statistically different CYP1A1 expression in lower dermal endothelial cells for bottlenose dolphin. Samples that were collected from animals between 38° and 41° latitude had less CYP1A1 expression than those collected at 35° latitude. If CYP1A1 expression were related to contaminants in these samples, as has been seen for other bottlenose dolphin (Chapter 4), these data would suggest that bottlenose dolphin have higher contaminant concentrations at lower latitudes along the US east coast.

Longitudinal gradients are inevitably more difficult to determine because the important information may not be longitude *per se*, but distance from shore, the presumed source of many ocean contaminants. Certainly, the only statistical difference in endothelial CYP1A1 expression (lower dermis) in bottlenose dolphin was between samples from 74° and 76° longitude. Mean CYP1A1 scores from 68° and 72° longitude were similar to or lower than those at 74° longitude, however differences in sample size and/or variation in CYP1A1 expression did not allow any differences to be detected statistically. It may be preferable to convert the longitude to distance from shore and repeat this analysis.

A difficulty in all of these geographic analyses is that these animals are highly mobile and without a clear understanding of the distances they travel, at least some, and possibly many samples will be inappropriately grouped based merely on where they were biopsied. Certainly, there is good agreement within these different analyses that bottlenose dolphin from areas near Cape Hatteras had higher levels of CYP1A1 expression. The Cape Hatteras animals had statistically greater CYP1A1 expression compared to the other regions and the Cape Hatteras region were the farthest south and west. The latitude analysis found the most southern groupings had statistically higher CYP1A1 expression in endothelial cells. Likewise, the most westerly longitudes had statistically higher endothelial CYP1A1 levels. Therefore, grouping the animals according to regions, latitude, or longitude all identified statistically higher CYP1A1 expression in bottlenose dolphin from the same general area.

In contrast to bottlenose dolphin, there were no differences in endothelial CYP1A1 expression, no matter how the data were examined. Two possibilities could explain this. First, the differences seen in bottlenose dolphin could be artifactual, possibly caused by inappropriately grouping samples, although these seems unlikely since dividing samples by region, latitude, or longitude resulted in the same conclusion. Second, there may be differences in the stock structure or range that these two species cover. It is possible that common dolphin move throughout the entire geographic area covered and therefore, represent a single stock within the Western Atlantic Ocean population.

Surprisingly, CYP1A1 levels in endothelial cells of offshore bottlenose dolphin were not significantly lower than that seen in coastal bottlenose dolphin (Chapter 4). CYP1A1 levels in both vascular smooth muscle and nerve cells were higher in the offshore animals. The reasons for this difference is unknown, particularly since contaminant concentrations in these offshore animals are unknown, making interpretation of CYP1A1 levels difficult. One possibility is that contaminant concentrations do not greatly decrease in offshore populations, or that the areas where animals were biopsied were not far enough offshore to see significant contaminant declines compared to coastal populations. Another possibility is that those animals with highest CYP1A1 expression utilize coastal habitats for feeding. Certainly, determining the distance offshore, instead of examining longitude alone, would be helpful to discern whether samples with higher CYP1A1 expression were closer to shore, and therefore more likely to have similar contaminant burdens as coastal populations. Lastly, the bottlenose dolphin sampled for this study were the offshore ecotype of bottlenose dolphin. These animals have morphological differences, namely they are much larger, than the coastal ecotype. There may be significant differences in prey choice and perhaps even some physiological differences between the ecotypes that would make them less comparable than we would currently expect.

Acknowledgments

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Table 1. Summary of Information on Bottlenose and Common Dolphin Biopsied for this Study. The total number of animals (percent of total samples) for each category are shown.

	Category	Bottlenose Dolphin	Common Dolphin
Age Class	Adult	138 (94)	84 (85)
	Intermediate	8 (5)	2 (2)
	Juvenile	0	0
	Unknown	1 (1)	13 (13)
Size Class	Large	120 (82)	81 (82)
	Medium	25 (17)	12 (12)
	Small	1 (1)	0
	Unknown	1 (1)	6 (6)
Total Biopsies		147	99

Table 2. CYP1A1 Expression in Various Cell Types from Integument Biopsies of Bottlenose and Common Dolphin. CYP1A1 expression was determined immunohistochemically and is scored on a scale of 0 to 15 (see text for details). Mean scores are shown (± standard deviation).

Cell Type	Bottlenose Dolphin	Common Dolphin
Vascular Endothelial - upper dermis	2.2 (±1.5)	2.8 (±1.8)
Vascular Endothelial - lower dermis	5.0 (±2.8)	6.2 (±2.4)
Vascular Smooth Muscle	5.2 (±1.9)	6.0 (±2.2
Peripheral Nerves	5.0 (±2.3)	3.9(±2.2)
Perineurium	0 (±0)	0.7 (±1.3)

Figure 1. Mean CYP1A1 expression in endothelial cells of the lower dermis from bottlenose and common dolphin, along a latitudinal gradient of the Western Atlantic Ocean. CYP1A1 expression was determined immunohistochemically and is scored in a range of 0 to 15 (see text for details). For bottlenose dolphin, those groups with different letters were found to be statistically different using an ANOVA and Scheffe Test, with $p<0.05$.

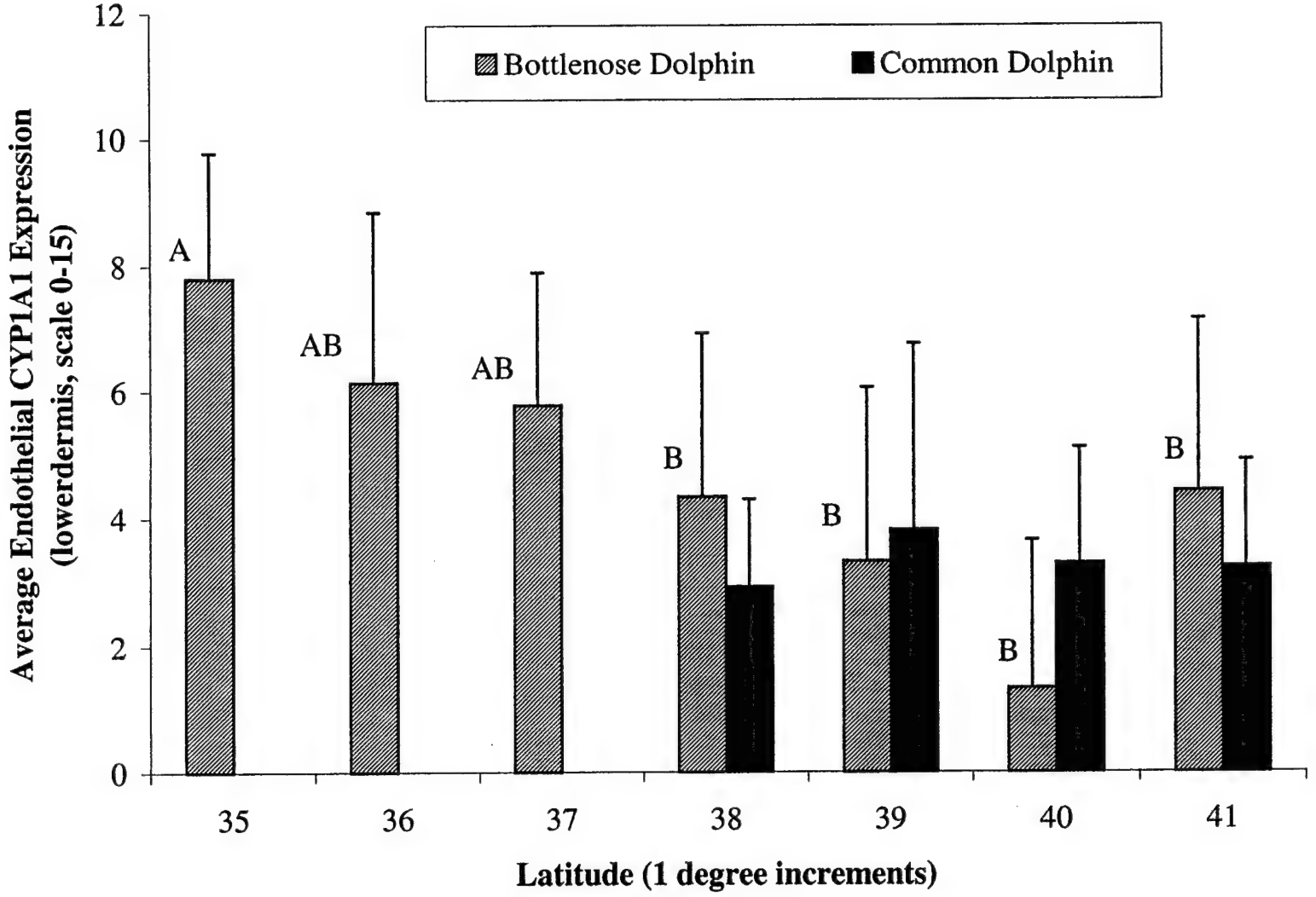
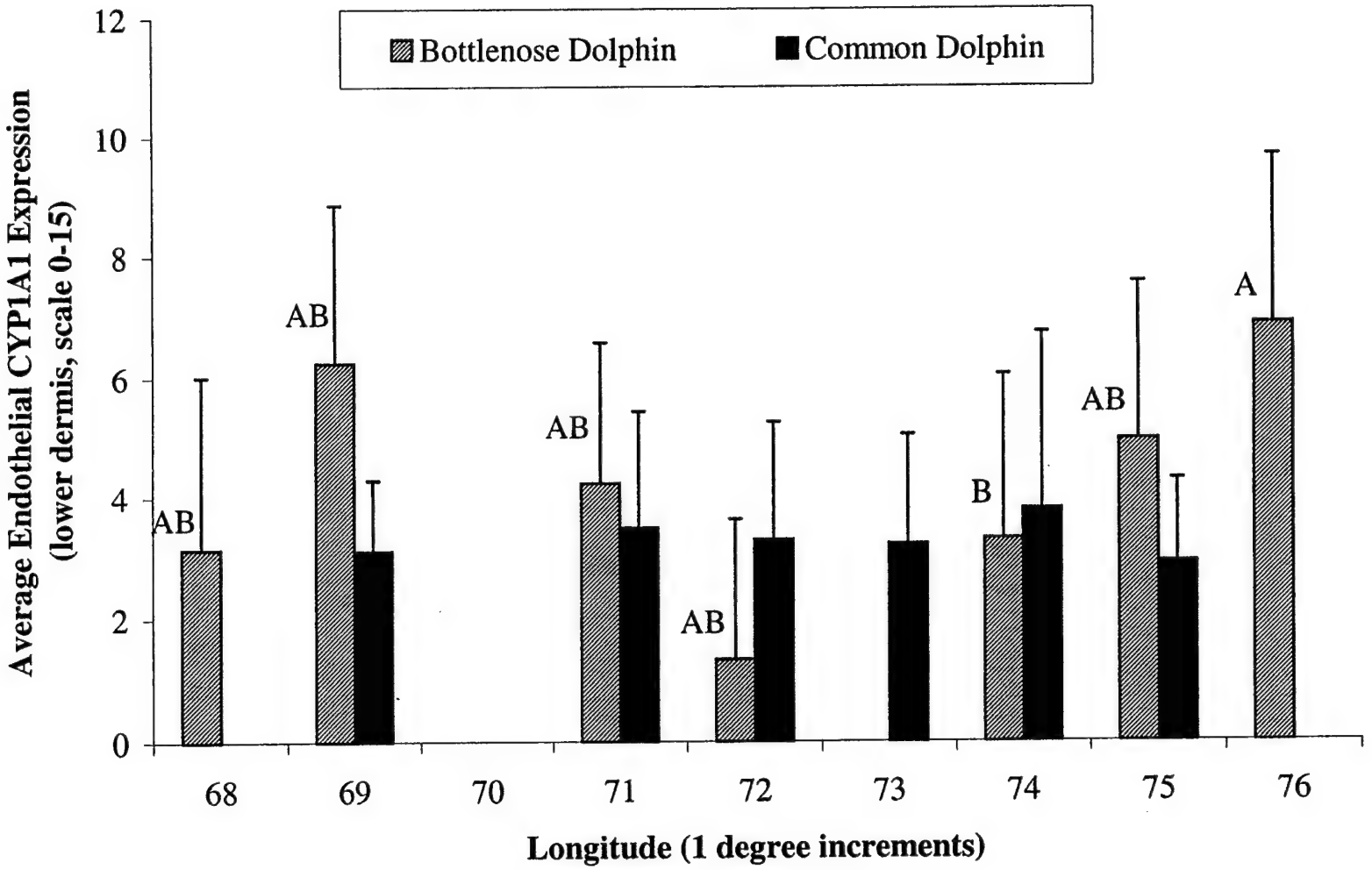

Bottlenose Dolphin
Common Dolphin
Average Endothelial CYP1A1 Expression (lowerdermis, scale 0-15)
12
10
8
6
4
2
0
A
AB
AB
B
B
B
B
35
36
37
38
39
40
41
Latitude (1 degree increments)

Figure 2. Mean CYP1A1 expression in endothelial cells of the lower dermis from bottlenose and common dolphin, along a longitudinal gradient of the Western Atlantic Ocean. CYP1A1 expression was determined immunohistochemically and is scored in a range of 0 to 15 (see text for details). For bottlenose dolphin, those groups with different letters were found to be statistically different using an ANOVA and Scheffe Test, with $p<0.05$.


Bottlenose Dolphin
Common Dolphin
Average Endothelial CYP1A1 Expression (lower dermis, scale 0-15)
12
10
8
6
4
2
0
AB
AB
AB
AB
B
AB
A
68
69
70
71
72
73
74
75
76
Longitude (1 degree increments)

Appendix 3. CYP1A1 is Not Inducible in Kidney and Lung Primary Cell Cultures from a Bottlenose Dolphin

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Abstract

Attempts at primary cell culture, particularly from liver, of cetaceans have proved relatively difficult. Two primary cell cultures from lung and kidney of bottlenose dolphin (*Tursiops truncatus*) were cultured and characterized. Endothelial cells were identified by selective labeling with dil-acetylated low density lipoproteins (dilAC-LDL). Both cell cultures were mixed populations, although the kidney cell culture contained primarily endothelial cells. The lung cell culture, which was mixed endothelial and epithelial cells, was grown in conditions that selected for endothelial cells. The two original cell cultures were tested for inducibility of cytochrome P450 1A1 (CYP1A1) by either β-napthoflavone (βNF) and/or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In each cell culture, CYP1A1 was either not induced or only marginally induced, indicating that these cell cultures are not useful for the determination of the dose-response relationship for CYP1A1 induction.

Introduction

The usefulness of cytochrome P450 1A1 (CYP1A1) induction as a biomarker of exposure and effects in cetaceans in hampered by our inability to directly determine the responsiveness of cetacean species to typical CYP1A1 inducers such as tetrachlorodibenzo-*p*-dioxin (TCDD), β- napthoflavone (βNF), and polychlorinated biphenyls (PCBs). These compounds have been used in many model or test organisms and direct comparison of the dose-response curve would allow us to directly determine the relative sensitivity of different species. Experimental exposures are precluded in cetacean species and other means of determining the responsiveness of these species are required. Knowledge of the dose-response relationships would also allow us to better interpret CYP1A1 data from environmentally exposed animals. Determining *in vitro* dose-response curves for multiple species could indicate the relative sensitivities of species for CYP1A1 induction.

Although induction of CYP1A1 by β-napthoflavone (βNF) has been demonstrated in manually cut skin and blubber biopsy samples from cetaceans (C. Godard, pers. com.),

we have yet to determine a complete dose response curve for any species, in any internal organ. Primary cell culture offers one of our best opportunities to determine dose-response curves for cetacean species. In rodents, the *in vivo* induction of CYP1A1 is similar to *in vitro* cell systems (Leece *et al.* 1985; Mason *et al.* 1986). We could expect that *in vitro* cell culture data would adequately model *in vivo* responses.

Primary culture of cetacean tissues has proved rather difficult, particularly for the liver. Several attempts at primary cell culture of a variety of internal organs have failed (J. Wilson, unpublished data and, Jensen 2000) and those that were successfully cultured have so far been non-responsive to typical CYP1A1 inducers (Jensen 2000). Through collaborations with Drs. R. Middlebrooks and R. Patterson, University of Southern Mississipppi, we have characterized and screened two primary cell lines from lung and kidney of bottlenose dolphin for inducibility of CYP1A1.

Materials and Methods

Cell Culture Conditions

Two primary cell cultures, generated from lung and kidney of a bottlenose dolphin, were generously donated by Dr. R. Middlebrooks, University of Southern Mississippi. Cells were cultured in M199 media (Sigma, St. Louis MO) with 10% cosmic calf serum (HyClone, Logan UT) at 37°C and 5% CO_2. Different culture conditions were originally tested with both lung and kidney cell culture and both cultures were found to grow well in several different medias (L-15, Dulbecco's MEM , and M199). Cells were split either 1:2 or 1:3 when cultures were confluent with 0.25% trypsin-EDTA solution (Sigma, St. Louis MO). Media was changed the day after cultures were split. Both cell cultures reached complete confluence after 2-3 days. When appropriate, cells culture aliquots were frozen in freezing media (MEM media, 10% calf serum, 10% DMSO), with a controlled rate of cooling (-1°C min^{-1}) in an isopropanol bath. Cultures were started from either passage 18 (lung) or 19 (kidney).

Identification of Cell Types and Selective Growth of Endothelial Cells

As with most primary cultures, both the lung and kidney lines were mixed cultures. Endothelial cells were identified by fluorescent microscopy, after incubating cells in M199 media with 20 μg ml^{-1} dil-acetylated low density lipoprotein (1,1'-dioctadecyl –3,3,3',3'-tetramethyl-indocarbocyanine perchlorate labeled acetylated LDL, dilAC-LDL, Biomedical Technology, Stoughton MA) at 37°C for four hours. Cells were washed with serum free media and viewed with fluorescence microscopy and TRITC long pass filters (excitation and emmission, 546 nm and 565 nm, respectively). Endothelial cells and macrophages are selectively labeled with dilAC-LDL, contrary to other cell types. Both the lung and kidney cell cultures contained endothelial cells, although the kidney cell line was predominantly endothelial cells and the lung was more of a mixed population (Figure 1). After lung cell cultures were trypsinized and removed from a flask, a small population of smaller, strongly adherent cells remained. These flasks were supplemented with fresh media and the remaining cells were cultured. This procedure enriched the number of endothelial cells in culture. After one or two rounds of selection, the lung cell culture was predominantly endothelial cells.

Inducibility of CYP1A1 in Different Cell Lines

Prior to plating for dosing experiments, cell densities were determined using 0.4% trypan blue (Sigma, St. Louis MO), according to manufacturer's directions. Cells were plated at varying densities, usually between 8 - 25 × 10^4 cells per well, in 48 well plates 24 hours prior to exposure. Cells were incubated in 0.5 ml M199 media with 1% cosmic calf serum for all dosing experiments. High serum content in cell culture media has been shown to decrease the effect of the inducer, by decreasing bioavailability and lowering the effective dose reaching the cell (Hestermann *et al.* 2000). Cells were exposed to varying concentrations of βNF (0.01, 0.1, 1, 3 and 10 μM) or TCDD (0.01, 0.1, 0.3, 1, 10, 15, 30 nM) dissolved in dimethylsulfoxide (0.5% well volume) for 48 hours. Kidney cell cultures were exposed to both inducer compounds, while lung cell cultures were exposed to βNF. The lung endothelial cell culture has not yet been tested for CYP1A1 inducibility. Ethoxyresorufin-O-deethylase (EROD) activity was determined according to

methods previously established for cells cultured in plates (Kennedy *et al.* 1995; Hahn *et al.* 1996). EROD activity was calculated as the pmoles of resorufin generated per mg of total protein per minute.

Results

CYP1A1 activity was not inducible by βNF, at concentrations up to 10 μM in either the lung or kidney cell culture (data not shown). Kidney cells were either not induced or marginally induced by TCDD (data not shown), but induction was so slight that it was not evident with all replicate experiments.

Discussion

These experiments indicate that the primary cell cultures tested, even those which were selectively grown for endothelial cells, are not inducible for CYP1A1 at doses that have been shown to induce CYP1A1 in other cell lines. Endothelial cells strongly express CYP1A1 in a number of internal organs, including lung, of beluga whale (see Chapter 2). Tubular epithelium and endothelial cells in kidney express CYP1A1 at a lower level than lung endothelia in beluga whale (see Chapter 2). CYP1A1 is inducible in both lung and kidney in other mammals (Omiecinski *et al.* 1990; Dey *et al.* 1999). The lung and kidney primary cell cultures were thought to be promising opportunities to assess the dose-response relationship of typical CYP1A1 inducers in cetacean cells. Unfortunately, we have been unable to generate primary cell cultures from liver, the organ that expresses the highest levels of CYP1A1. Even so, ongoing efforts to culture cetacean tissues and generate pure cultures may eventually identify cell lines which are responsive to typical CYP1A1 inducers and give us an *in vitro* system to assess dose-response relationships for cetacean species.

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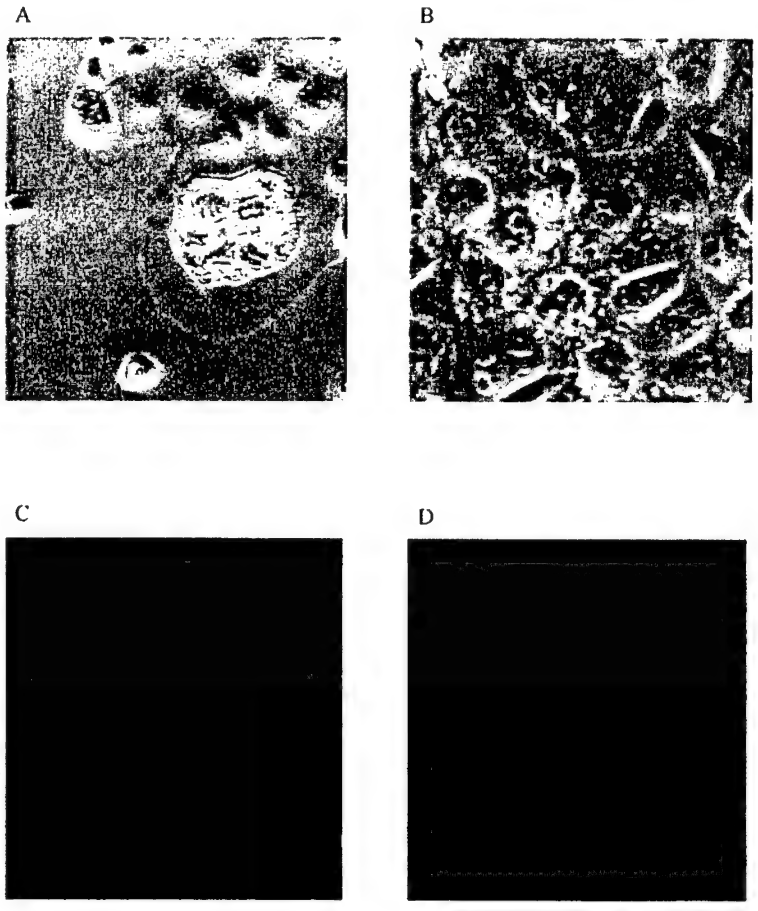
Figure 1. Normal and dilAC-LDL labeled primary cell cultures from lung and kidney of bottlenose dolphin. Microscopy images of primary cell cultures (A and B) prior and (C and D) after dilAC-LDL labeling of endothelial cells. Panels A and C show lung cultures and panels B and D show kidney cultures. See text for explanation of dilAC-LDL labeling.


A
B
C
D

Appendix 4. Supplementary Data for Chapter 2

Table 1. CYP1A1 Expression in Internal Organs from Beluga Whale from the Arctic (Mackenzie Delta and Hudson Bay) and the St. Lawrence Estuary. Data shown was collected by immunohistochemistry, data is shown only for cell types that stained for CYP1A1 expression with the monoclonal antibody 1-12-3 in at least one individual. CYP1A1 expression is scored on a scale of 0-15. Data shown is mean expression for males and females from each location.

Organ	Cell Type	Mackenzie Delta		Hudson Bay		St. Lawrence Estuary	
		M	F	M	F	M	F
Heart	Endothelia	-	-	1.3 ±1.7 (9)	3.0 ±0 (3)	-	-
Heart	Epicardium	-	-	0.8 ±1.7 (9)	0.0 (1)	-	-
Cerebrum	Endothelia	1.6 ±1.4 (12)	1.5 ±2.1 (2)	1.7 ±2.6 (6)	0.0 ±0 (2)	2.3 ±3.7 (8)	3.0 ±3.0 (5)
Cerebrum	Vascular Smooth Muscle	4.7 ±1.6 (12)	1.5 ±1.7 (2)	0.0 ±0 (6)	0.0 ±0 (2)	0.0 ±0 (8)	0.0 ±0 (5)
Cerebrum	Neuron	4.8 ±1.9 (12)	3.5 ±0.7 (2)	0.0 ±0 (6)	0.0 ±0 (2)	0.0 ±0 (8)	0.2 ±0.4 (5)
Cerebrum	Neuroglia	3.0 ±1.5 (12)	2.0 ±1.4 (2)	0.8 ±2 (6)	0.0 ±0 (2)	0.8 ±1.8 (8)	2.2 ±2.5 (5)
Skin[1]	Endothelia	5.5 ±1.5 (11)	3.5 ±0.7 (2)	-	-	-	-
Skin	Vascular Smooth Muscle	3.8 ±0.6 (11)	3.0 ±1.4 (2)	-	-	-	-
Skin	Peripheral Nerve	2 (1)	*	-	-	-	-
Lung	Endothelia	9.1 ±2.1 (12)	8.0 ±2.8 (2)	6.7 ±3.7 (9)	5.5 ±1.8 (3)	7.0 ±1.4 (4)	6.3 ±3.5 (5)
Lung	Vascular Smooth Muscle	5.6 ±1.1 (12)	4.0 ±0 (2)	0.0 ±0 (9)	0.0 ±0 (3)	0.0 ±0 (4)	0.0 ±0 (5)
Lung	Bronchial Epithelia	4.0 (1)	*	0.5 ±1.5 (9)	0.0 ±0 (3)	0.0 ±0 (4)	0.0 ±0 (5)
Lung	Chondrocytes	3.5 ±1.9 (12)	0.5 ±0.7 (2)	0.6 ±1.1 (9)	0.0 ±0 (3)	0.0 ±0 (4)	0.0 ±0 (5)
Colon	Endothelia	5.6 ±2.6 (5)	-	-	-	-	-
Colon	Muscularis Mucosae	6.3 ±2 (5)	-	-	-	-	-
Colon	Goblet Cells	0.4 ±0.9 (5)	-	-	-	-	-
Colon	Absorptive Cells	0.6 ±1.3 (5)	-	-	-	-	-
Colon	Peripheral Nerve	5 ±1.2 (4)					
Liver	Endothelia	3.8 ±3.1 (12)	2.0 ±2.8 (2)	2.9 ±4.1 (7)	0.7 ±1.2 (3)	0.0 ±0 (8)	1.6 ±1.9 (6)

Table 1. Continued

Organ	Cell Type	Mackenzie Delta		Hudson Bay		St. Lawrence Estuary	
		M	F	M	F	M	F
Liver	Hepatic parenchyma	11.7 ±2 (12)	10.0 ±2.8 (2)	12.4 ±2.1 (7)	10.0 ±3.5 (3)	0.3[4] ±0.8 (8)	3.3[4] ±3.2 (6)
Liver	Bile Collecting Duct Epithelia	0.0 ±0 (12)	0.0 ±0 (2)	0.0 ±0 (7)	1.0 ±1.7(3)	0.5 ±1.2 (8)	1.0 ±2.4 (6)
Kidney	Endothelia	6.5[4] ±2.1 (12)	9.0[4] ±1.4 (2)	1.8 ±2.3 (9)	0.0 ±0 (3)	0.0 ±0 (7)	2.2 ±2.8 (7)
Kidney	Vascular Smooth Muscle	4.9 ±1.3 (12)	4.0 ±0 (2)	0.0 ±0 (9)	0.0 ±0 (3)	0.0 ±0 (7)	0.0 ±0 (7)
Kidney	Renal Corpuscle	1.3 ±1.2 (12)	0.0 ±0 (2)	0.0 ±0 (9)	0.0 ±0 (3)	0.0 ±0 (7)	0.0 ±0 (7)
Kidney	Tubular Epithelia - Cortex	8.9[4] ±0.4 (12)	8.3[4] ±1.1 (2)	0.4 ±1.3 (9)	0.0 ±0 (3)	0.0 ±0 (7)	0.9 ±2.3 (7)
Kidney	Tubular Epithelia - Medulla	3.6 ±1.3 (12)	1.8 ±0.4 (2)	1.2 ±1.8 (9)	1.0 ±0 (3)	0.0 ±0 (7)	0.0 ±0 (7)
Bladder	Endothelia	8.0 ±1.2 (7)	-	-	0.0 (1)	7.6 ±6.1 (6)	13.1 ±3.8 (4)
Bladder	Smooth Muscle of the Bladder	6.3 ±0.9 (7)	-	-	6.0 (1)	6.0 ±2.4 (6)	5.3 ±3.8 (4)
Bladder	Transitional Epithelia	8.0 ±1.2 (7)	-	-	12.0 (1)	6.0 ±8.5 (6)	11.3 ±2.9 (4)
Adrenal gland	Endothelia	7.1 ±2.5 (9)	8.3 ±6 (2)	-	-	-	3.0 ±4.2 (2)
Adrenal gland	Vascular Smooth Muscle	5.7 ±0.8 (9)	2.5 ±0.7 (2)	-	-	-	0.0 ±0 (2)
Adrenal gland	Cortex	6.8 ±1.7 (9)	5.0 ±0 (2)	-	-	-	5.0 ±1.4 (2)
Adrenal gland	Medulla	1.4 ±1.5 (9)	0.0 ±0 (2)	-	-	-	6.5 ±4.9 (2)
Thyroid	Endothelia	-	-	-	-	-	10.5 ±6.4 (2)
Ovary	Endothelia		2.3 ±3.2 (2)		-		1.3 ±1.5 (4)
Ovary	Primordial Follicles		*		-		3.3 ±1.9 (4)
Ovary	Luteal Cells[2]		3.0 ±4.2 (2)		-		*
Ovary	Medulla		*		-		2.5 ±0.6 (4)
Testes	Endothelia	5.0 ±2.6 (12)		1.0 ±1.4 (2)		2.7 ±4.6 (4)	
Testes	Vascular Smooth Muscle	5.6 ±1.7 (12)		0.0 ±0 (2)		0.0 ±0 (4)	
Testes	Spermatogenic Series[3]	6.3 ±1.5 (12)		3.0 ±1.4 (2)		4.7 ±1.2 (4)	
Testes	Leydig Cells	3.3 ±1.5 (12)		1.0 ±1.4 (2)		0.0 ±0 (4)	

- organ not available, * cell type not present in section examined, [1] Skin samples from 3 adult captive beluga showed no CYP1A1 staining in any cell type, [2] corpus luteum of pregnancy, [3] primarily spermatogonia and primary spermatocytes, may include some Sertoli cells, [4] significantly different from animals from other sites at $p<0.05$.

Table 2. CYP1A1 Expression in Internal Organs from a Beluga Whale Neonate. Data shown was collected by immunohistochemistry, data is shown only for cell types that expressed CYP1A1. CYP1A1 expression is scored on a scale of 0-15. This animal died 7 minutes after birth in a captive facility. The animal identification number is 94-457.

Organ	Cell Type	CYP1A1 Expression
Heart	Endothelia	3
Heart	Endothelia of Epicardium	6
Heart	Mesothlial Cells of Epicardium	3
Heart	Leucocytes[2]	8
Cerebrum	Endothelia	4
Cerebrum	Neuron	2
Muscle	Macrophages	3
Lung	Endothelia	8
Forestomach	Endothelia	3
Forestomach	Leucocytes	3
Forestomach	Epithelia	3
Stomach[1]	Endothelia	8
Stomach	Leucocytes	3
Small Intestine	Endothelia	4
Small Intestine	Leucocytes	3
Liver	Endothelia	3
Liver	Hepatic Parenchyma	4
Pancreas	Endothelia	1
Kidney	Endothelia	8
Kidney	Tubular Epithelia - Cortex	6
Adrenal	Leucocytes	6
Adrenal	Endothelia	4
Spleen	No staining in any cell type	-
Thymus	No staining in any cell type	-

[1]The cetacean stomach is a diverticulated composite stomach with 4 chambers. This sample was not taken by the forestomach, but from one of the other chambers. [2] Leucocytes staining for CYP1A1 consisted primarily of macrophages in this animal.

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16. Abstract (Limit: 200 words)

Contaminant exposure has been associated with toxicity in cetaceans. Early biochemical responses prior to, but associated with, contaminant-induced toxicity will aid in understanding the susceptibility of cetaceans to contaminants. Cytochrome P450 1A1 (CYP1A1) induction by certain polychlorinated biphenyls (PCBs) has been correlated with toxicity in rodents. CYP1A1 expression was measured in organs from beluga whales and white-sided dolphins and integument biopsies from bottlenose dolphins. Age and sex do not influence CYP1A1 expression in these species. Mono-*ortho* PCB concentrations were correlated with CYP1A1 in bottlenose and white-sided dolphin. White-sided dolphins have higher PCB concentrations but lower hepatic CYP1A1 expression than beluga. Beluga thus appear sensitive to CYP1A1 induction by PCBs. These studies demonstrate that comparative studies between cetacean species could determine the relative susceptibility of cetaceans.

Aromatase is responsible for estrogen production and studies suggest that PCBs may alter its activity. The sequence and catalytic properties of a cetacean aromatase were similar to other mammalian aromatases. Evolutionary analyses of vertebrate aromatases revealed striking functional conservation. These data show that aromatase studies in other mammals are applicable to cetacean species. Experimental work in model organisms, coupled with studies in cetaceans, could infer the physiological role and transcriptional control of cetacean aromatase.

17. Document Analysis **a. Descriptors**
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polychlorinated biphenyl

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